

REMARKS

Claims 1-52 were pending. Claims 1-13, 16, 18, 21, 26, 28, 32-33, 37-42, 44 and 48-50 are canceled. Claims 14-15, 17, 19-20, 23-25, 27, 29, 30, 34-36, 43, 45, 46, and 51 have been amended. Claims 52-55 are new, and find support in originally filed claims 12-13. No new matter is added. Applicants respectfully request reconsideration of the rejections, and allowance of the presently pending claims, 14-15, 17, 19-20, 22-25, 27, 29-31, 34-36, 43, 45-47, and 51-55.

Claims 3, 17, 23-36 and 51 have been rejected under 35 U.S.C. 112, second paragraph as indefinite in the recitation of the terms "part of a candidate gene", "portion of a gene", and "can be". The claims have been amended to clarify the subject matter and to recite definite terms. Withdrawal of the rejection is requested.

Claims 1-4, 14-18, 23-26, 30, 33, 34, 35, 36, 38, 39, 41 and 48-50 have been rejected under 35 U.S.C. 102 as being anticipated by Leptin, U.S. Patent no. 6,135,942. Applicants respectfully submit that the present claims are not anticipated by Leptin. The present claims are drawn to the use of dsRNA in the specific attenuation of expression of genes in mammalian neural cells. Leptin discloses the genes of *Drosophila melanogaster*, and does not teach methods utilizing mammalian neural cells. Withdrawal of the rejection is requested.

Claims 5-13, 19-21 and 37 have been rejected under 35 U.S.C. 103 as unpatentable over Leptin, in view of Petryshyn. Claim 22 has been rejected under 35 U.S.C. 103 as unpatentable over Leptin, in view of Petryshyn, and further in view of Kreitman. Claims 31, 32, 40 and 42-44 have been rejected under 35 U.S.C. 103 as unpatentable over Leptin, in view of Der *et al.*, U.S. Patent no. 6,077,686. Claims 45-47 and 51 have been rejected under 35 U.S.C. 103 as unpatentable over Leptin, in view of Der *et al.*, and further in view of Staddon *et al.* (U.S. Patent no. 6,312,686). Applicants respectfully submit that the presently claimed invention is not made obvious by the cited combinations of references.

The claims have been amended to recite methods using a dsRNA that comprises at least 100 nucleotides of sequence identity with a candidate gene, in order to validate the function of the candidate gene in a mammalian neural cell by specific attenuation of that gene. The prior art cited by the Examiner teaches the use of double stranded RNA interference in insect cells. However, the effect of such RNAs in mammalian cells has been very different from what is observed in the prior art systems. Applicants have attached herewith a research article published by co-inventors Li Gan,

Kristen Anton and Mirella Gonzalez-Zulueta (Gan *et al.* (2002) J. Neuroscience Methods **121**:151-157), which discusses the unexpected findings of the present invention.

Specific gene silencing mediated by double stranded RNA has been used in diverse invertebrate systems, and RNA species of about 25 nucleotides have been found to silence gene expression in plants. The interfering RNA in these systems has been shown to be a small inhibitory RNA (siRNA) of about 21-25 nucleotides in length.

However, the prior art teaches that vertebrate cells respond to dsRNA longer than about 30 nucleotides by inducing interferon alpha and beta expression. The effect of this interferon expression is a general inhibition of protein synthesis and non-specific degradation of mRNA, in contrast to the presently claimed **specific** attenuation of mRNA activity. For example, see the attached review paper by Stark *et al.* (1998) Annu. Rev. Biochem. **67**:227-264. This paper states (page 255) that “it has long been recognized that a combination of IFN and dsRNA is cytotoxic”.

A number of publications have addressed the induction of interferon synthesis by double stranded RNA, and the involvement of the double-stranded-RNA dependent kinase PKR into mediating interferon activity. For example, see the attached research articles Der and Lau (1995) P.N.A.S. **92**:8841-8845, which demonstrates the role of double stranded RNA in the induction of expression and functional effects of interferon.

The attached article by Elbashir *et al.* (2001) Nature **411**:494-498, states that “it has not been possible to detect potent and specific RNA interference in commonly used mammalian cell culture systems . . . applying dsRNA that varies in size between 38 and 1,662 base pairs”. The author goes on to state that “it is known that dsRNA in the cytoplasm of mammalian cells can trigger profound psychological reactions that lead to the induction of interferon synthesis . . . these responses are **intrinsically non-specific** to the inducing dsRNA” (emphasis added). Elbashir *et al.* then describe the use of small double stranded RNAs in the attenuation of expression, thus teaching one of skill in the art that (a) large dsRNA leads to non-specific attenuation and interferon expression and that (b) small dsRNAs are required for this method to be effective in mammalian cells.

It would have been expected by one of skill in the art that the use of dsRNA of at least 100 nt in length would lead to interferon expression in neuronal cells. As demonstrated by Ward *et al.* (1995) J. Neuroimmunology **58**:145-155 (attached), “in primary neuronal cultures following exposure of these cells to known IFN-inducing agents, including double-stranded RNA . . . it was found that neurons rapidly express high levels of IFN- β .”

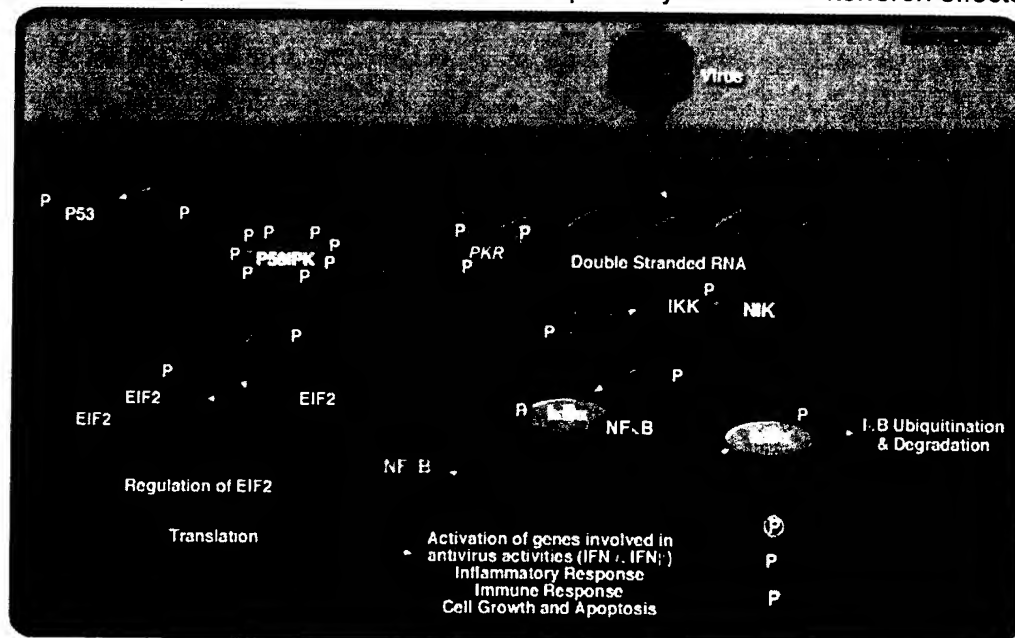
One of skill in the art would not have had a reasonable expectation that dsRNA of at least 100 nt. in length would result in specific attenuation of expression. And yet, as shown by Applicants, (page 56, last paragraph,ff) specific attenuation was observed in neuronal cells for GFP (green

fluorescent protein), and for PARP, both in the levels of the protein produced, and in the functional effects mediated by the protein. Cells transfected with dsPARP-N showed significant protection against OGD-induced cell death compared with mock-transfected cells and those transfected with dsGFP. These results confirmed the essential role of PARP as a mediator in ischemia-induced neuronal damage, and validate the use of RNAi in the analysis of the role of novel and known genes in neurons.

Leptin, U.S. 6,135,942, has been cited against the present claims under 35 U.S.C. 102 and 103. As discussed above, the present invention is drawn to the use of dsRNA of at least 100 nt, in mammalian neural cells. The teachings of Leptin are specific to *Drosophila* cells. Because *Drosophila* cells do not share the biology of mammalian cells with respect to induction of interferon by dsRNA, Leptin does not teach or suggest the function of RNAi in mammalian cells.

Petryshyn, U.S. 6,124,091, has been cited in combination with Leptin. Applicants respectfully submit that Petryshyn does not suggest the present invention, alone or in combination with Leptin. As stated by Petryshyn, column 3, lines 7-15, the patent provides for "a single and specific cellular R-RNA which, when transcribed in vitro, gives rise to an RNA transcript which retains its property to **activate PKR** . . . [and] a 226-252 nucleotide fragment of the partial cDNA which corresponds to that portion of R-RNA necessary for PKR activation." This statement supports Applicants discussion (above) of interferon induction. As shown in the attached paper by Der and Lau, double stranded RNA induces interferon expression, and the double-stranded-RNA dependent kinase **PKR** into mediating interferon activity. Therefore, Petryshyn teaches an RNA that has the **expected** activity of activating PKR.

The following figure demonstrates the cellular pathways active in interferon effects:



Applicants respectfully submit that it was well known in the art that dsRNA activates PKR, and that such an effect teaches away from the specific attenuation observed by Applicants.

The citation in the Office Action of Example 1 of Petryshyn as showing dsRNA attenuation of expression of a candidate gene is not understood. Example 1 of Petryshyn discloses the cDNA synthesis of R-RNA, and does not disclose RNA interference. If the cite was intended to be Example 11 of Petryshyn, Applicants respectfully submit that the use of short anti-sense oligonucleotides, which are single stranded (noted at col. 28, line 65 as selected for “minimal self-folding”) does not suggest the presently claimed invention. As explained above, the effects of short RNAs are dramatically different than long RNAs, because of the effect on interference induction.

Further the effects of single stranded oligonucleotides are distinct from that of double stranded RNA, and one does not predict the activity of the other. As discussed in the attached review of RNA interference (Hannon (2002) Nature **418**:244-51), “The phenomenon of RNAi was first discovered in the nematode worm *Caenorhabditis elegans* as a response to double-stranded RNA (dsRNA), which resulted in sequence-specific gene silencing. Following on from the studies of Guo and Kemphues, who had found that **sense RNA was as effective as antisense RNA** for suppressing gene expression in worms, Fire, Mello and colleagues were attempting to use antisense RNA as an approach to inhibit gene expression. Their breakthrough was to test the synergy of sense and antisense RNAs, and they duly found that **the dsRNA mixture was at least tenfold more potent as a silencing trigger than were sense or antisense RNAs** alone.” Therefore, it is recognized in the art that dsRNAs silencing differs dramatically from anti-sense inhibition.

Applicants respectfully submit that Petryshyn does not make obvious the presently claimed invention, and does not remedy the deficiencies of the primary reference, Leptin.

The citation of Kreitman *et al.* is provided for teaching the use of the restriction enzyme RsaI. Applicants respectfully submit that the use of the restriction enzyme is not relied upon for patentability, but is cited as one embodiment of the invention. Kreitman *et al.* does not remedy the deficiencies of the primary reference, which fails to teach or suggest the use of dsRNA of greater than 100 nt in neuronal cells to specifically attenuate gene expression.

Similarly, Villeponteau *et al.* fail to remedy the deficiencies of the primary reference by failing to teach the use of dsRNA of greater than 100 nt in neuronal cells to specifically attenuate gene expression. Applicants note that Villeponteau *et al.* describe the generation of subtractive libraries, but do not teach the normalization of the library. Normalized libraries allow for the isolation of rare transcripts that are not otherwise obtainable.

Der et al. suggests the use of anti-sense oligonucleotides in neuronal cells, but fails to teach the use of dsRNA. As discussed above, the effects of RNA silencing, or interference, which result from the introduction of specific double stranded RNA, are very different from the activities of short, single stranded oligonucleotides. One of skill in the art is not informed of the present invention by the teachings of *Der* combined with *Leptin*.

Staddon et al. is directed to alterations in permeability of tight junctions in epithelial and endothelial cells, but does not teach the use of long dsRNA to attenuate gene expression.

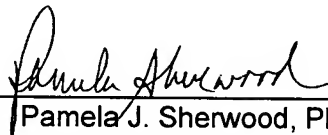
In view of the above amendments and remarks, Applicants respectfully submit that the present invention meets the requirements of 35 U.S.C. 102 and 103. Withdrawal of the rejections is requested.

Applicants submit that all of the claims are now in condition for allowance, which action is requested. If the Examiner finds that a Telephone Conference would expedite the prosecution of this application, he is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any other fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815, order number AGYT-013CIP.

Respectfully submitted,

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RNA interference

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A conserved biological response to double-stranded RNA, known variously as RNA interference (RNAi) or post-transcriptional gene silencing, mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids, and regulates the expression of protein-coding genes. RNAi has been cultivated as a means to manipulate gene expression experimentally and to probe gene function on a whole-genome scale.

The phenomenon of RNAi was first discovered in the nematode worm *Caenorhabditis elegans* as a response to double-stranded RNA (dsRNA), which resulted in sequence-specific gene silencing¹. Following on from the studies of Guo and Kemphues, who had found that sense RNA was as effective as antisense RNA for suppressing gene expression in worms², Fire, Mello and colleagues¹ were attempting to use antisense RNA as an approach to inhibit gene expression. Their breakthrough was to test the synergy of sense and antisense RNAs, and they duly found that the dsRNA mixture was at least tenfold more potent as a silencing trigger than were sense or antisense RNAs alone¹. Silencing by dsRNAs had a number of remarkable properties — RNAi could be provoked by injection of dsRNA into the *C. elegans* gonad or by introduction of dsRNA through feeding either of dsRNA itself or of bacteria engineered to express it³. Furthermore, exposure of a parental animal to only a few molecules of dsRNA per cell triggered gene silencing throughout the treated animal (systemic silencing) and in its F₁ (first generation) progeny (Fig. 1).

From this discovery emerged the notion that a number of previously characterized, homology-dependent gene-silencing mechanisms might share a common biological root. Several years previously, Richard Jorgensen had been engineering transgenic petunias with the goal of altering pigmentation. But introducing exogenous transgenes did not deepen flower colour as expected. Instead, flowers showed variegated pigmentation, with some lacking pigment altogether (refs 4, 5, and reviewed in ref. 6). This indicated that not only were the transgenes themselves inactive, but also that the added DNA sequences somehow affected expression of the endogenous loci. This phenomenon, called co-suppression, can be produced by highly expressed, single-copy transgenes^{7,8} or by transgenes, expressed at a more modest level, that integrate into the genome in complex, multicopy arrays⁹. In parallel, several laboratories found that plants responded to RNA viruses by targeting viral RNAs for destruction^{10–13}. Notably, silencing of endogenous genes could also be triggered by inclusion of homologous sequences in a virus replicon.

What is clear in retrospect is that both complex transgene arrays and replicating RNA viruses generate dsRNA. In plant systems, dsRNAs that are introduced from exogenous sources or that are transcribed from engineered inverted repeats are potent inducers of gene silencing (reviewed in ref. 14). But co-suppression phenomena are not restricted to plants: similar outcomes have been noted in unicellular organisms, such as *Neurospora*, and in metazoans, such as

Drosophila, *C. elegans* and mammals^{15–18}. In a few cases, silencing has been correlated with integration of transgenes as complex arrays that can produce dsRNA directly, although silencing can also be triggered by the presence of single-copy or dispersed elements¹⁸. What remains a mystery is how, and indeed whether, such elements produce the dsRNA silencing trigger that has become a hallmark of RNAi. It has been proposed that endogenous RNA-directed RNA polymerases (RdRPs) may recognize 'aberrant transcripts' derived from highly expressed loci and convert these into dsRNA¹⁹. Indeed, homologues of these enzymes have proven essential for silencing in *C. elegans*, fungi and plants, and this is discussed below.

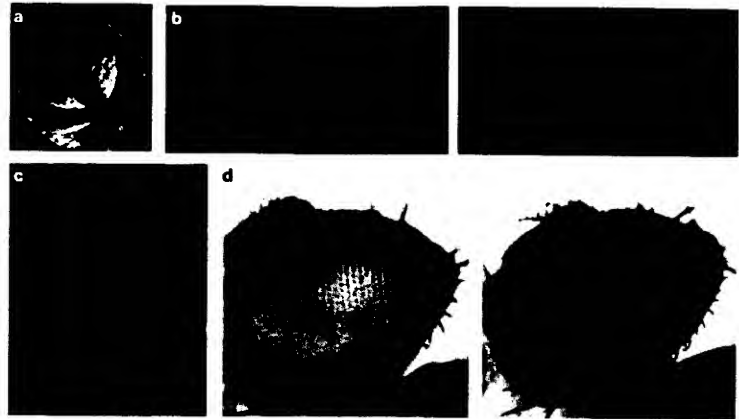
Genetic and biochemical studies have now confirmed that RNAi, co-suppression and virus-induced gene silencing share mechanistic similarities, and that the biological pathways underlying dsRNA-induced gene silencing exist in many, if not most, eukaryotic organisms (Fig. 1). What are the mechanisms by which dsRNAs induce silencing of homologous sequences, either exogenous or endogenous? What are the biological functions of these processes? And how are they related in evolutionarily divergent fungi, plants and animals?

Silencing machinery operates at multiple levels

In *C. elegans*, initial observations were consistent with dsRNA-induced silencing operating at the post-transcriptional level. Exposure to dsRNAs resulted in loss of corresponding messenger RNAs (mRNAs), and promoter and intronic sequences were largely ineffective as silencing triggers¹. A post-transcriptional mode was also consistent with data from plant systems in which exposure to dsRNA²⁰, for example in the form of an RNA virus, triggered depletion of mRNA sequences without an apparent effect on the rate of transcription²¹. Indeed, viral transcripts themselves were targeted, despite the fact that these were synthesized cytoplasmically by transcription of RNA genomes¹⁰. These studies led to the notion that RNAi induced degradation of homologous mRNAs, and this hypothesis has been validated by biochemical analysis.

But the RNAi machinery affects gene expression through additional mechanisms. In plants, exposure to dsRNA induces genomic methylation of sequences homologous to the silencing trigger²². If the trigger shares sequence with a promoter, the targeted gene can become transcriptionally silenced²³. Recent studies have suggested that the RNAi machinery may also affect gene expression at the level of chromatin structure in *Drosophila*, *C. elegans* and fungi (refs 18, 24–26, and R. Martienssen, T. Volpe, I. Hall and S. Grewal, unpublished data). Finally, in *C. elegans*, endogenously

Figure 1 Double-stranded RNA can be introduced experimentally to silence target genes of interest. In plants, silencing can be triggered, for example, by engineered RNA viruses or by inverted repeat transgenes. In worms, silencing can be triggered by injection or feeding of dsRNA. In both of these systems, silencing is systemic and spreads throughout the organism. **a**, A silencing signal moves from the veins into leaf tissue. Green is green fluorescent protein (GFP) fluorescence and red is chlorophyll fluorescence that is seen upon silencing of the GFP transgene. **b**, *C. elegans* engineered to express GFP in nuclei. Animals on the right have been treated with a control dsRNA, whereas those on the left have been exposed to GFP dsRNA. Some neuronal nuclei remain fluorescent, correlating with low expression of a protein required for systemic RNAi⁵⁹. **c**, HeLa cells treated with an ORC6 siRNA and stained for tubulin (green) and DNA (red). Depletion of ORC6 results in accumulation of multinucleated cells. Stable silencing can also be induced by expression of dsRNA as hairpins or snap-back RNAs. **d**, Adult *Drosophila* express a hairpin homologous to the white gene (left), which results in unpigmented eyes compared with wild type (right).



encoded inducers of the RNAi machinery (for example, *lin-4*) operate at the level of protein synthesis²⁷. Although translational control by dsRNA has not been established definitively in other systems, the conservation of *let-7* and related RNAs²⁸ suggests that this regulatory mode may be a further common mechanism through which RNAi pathways control the expression of cellular genes.

Mechanism of post-transcriptional gene silencing

Our present understanding of the mechanisms underlying dsRNA-induced gene silencing is derived from genetic studies in *C. elegans* and plants and from biochemical studies of *Drosophila* extracts. In the latter case, Carthew and colleagues laid the foundations by showing that injection of dsRNA into *Drosophila* embryos induced sequence-specific silencing at the post-transcriptional level²⁹. Sharp and colleagues then tested the possibility that *Drosophila* embryo extracts, previously used to study translational regulation, might be competent for RNAi³⁰. Incubation of dsRNA in these cell-free lysates reduced their ability to synthesize luciferase from a synthetic mRNA. This correlated with destabilization of the mRNA and suggested that dsRNA might bring about silencing by triggering the assembly of a nuclease complex that targets homologous RNAs for degradation.

This effector nuclease, now known as RISC (RNA-induced silencing complex), was isolated from extracts of *Drosophila* S2 cells in which RNAi had been triggered by treatment with dsRNA *in vivo*³¹. A key question was how this complex might identify cognate substrates. Fire and Mello had originally proposed that some derivative of the dsRNA would guide the identification of substrates for RNAi, and the first clue in the hunt for such 'guide RNAs' came from the study of silencing in plants. Hamilton and Baulcombe³² sought antisense RNAs that were homologous to genes being targeted by co-suppression. They found a ~25-nucleotide RNA that appeared only in plant lines containing a suppressed transgene, and found that similar species appeared during virus-induced gene silencing. Similar small RNAs were produced from dsRNAs in *Drosophila* embryo extracts³³, and partial purification of the RISC complex showed that these small RNAs co-fractionated with nuclease activity³¹.

These findings forged a link between transgene co-suppression in plants and RNAi in animals. In addition, a model for RNAi and related silencing phenomenon began to emerge (Fig. 2). According to this model, initiation of silencing occurs upon recognition of dsRNA by a machinery that converts the silencing trigger to ~21–25-nucleotide RNAs. These small interfering RNAs (siRNAs) are a signature of this family of silencing pathways and, by joining an effector complex RISC, they guide that complex to homologous substrates.

This convergence of observations from diverse experimental systems suggested that a conserved biochemical mechanism would lie at the core of homology-dependent gene-silencing responses.

However, the varied biology of dsRNA-induced silencing — for example, the heritable and systemic nature of silencing in *C. elegans* compared to apparently cell-autonomous, non-heritable silencing in *Drosophila* and mammals — suggested that this core machinery probably adapted to meet specific biological needs in different organisms.

The initiation step

The model outlined in Fig. 2 implies that the dsRNA silencing trigger is cleaved to produce siRNAs. Support for this emerged first from studies of *Drosophila* embryo extracts, which contained an activity capable of processing long dsRNA substrates into ~22-nucleotide fragments³³. Analysis of these RNAs showed that they were double stranded and contained 5'-phosphorylated termini^{33,34}. The quest for the enzyme that initiates RNAi led to the RNase III ribonuclease family, which displays specificity for dsRNAs and generates such termini.

RNase III enzymes can be divided into three classes based upon domain structure: bacterial RNase III contains a single catalytic domain and a dsRNA-binding domain; Drosha family nucleases contain dual catalytic domains³⁵; and a third family also contains dual catalytic domains and additional helicase and PAZ motifs³⁶. Members of this third class of RNases were found to process dsRNA into siRNAs and were therefore proposed to initiate RNAi³⁶. This family, now named the Dicer enzymes, are evolutionarily conserved, and proteins from *Drosophila*, *Arabidopsis*, the insect *Spodoptera frugiperda*, tobacco, *C. elegans*, mammals and *Neurospora* have all been shown to recognize and process dsRNA into siRNAs of a characteristic size for the relevant species (refs 36, 37, and A. M. Denli and G.J.H., unpublished data). Genetic evidence has also emerged from *C. elegans* and *Arabidopsis* that is consistent with Dicer acting in the RNAi pathway: Dicer is required for RNAi in the *C. elegans* germline^{37–39}, and a hypomorphic allele of *Carpel Factory* can intensify the phenotypes of weak *Argonaute-1* alleles in *Arabidopsis* (C. Kidner and R. Martienssen, personal communication).

Recently, the structure of an RNase III catalytic domain has led to a model for the generation of ~22-nucleotide RNAs by Dicer cleavage⁴⁰ (Fig. 2). It is thought that bacterial RNase III functions as a dimeric enzyme and, in the structural model, antiparallel RNase III domains produce two compound catalytic centres, each of which is formed by contributions from both monomers. The sequences of Dicer and Drosha RNase III domains reveal deviations from the consensus in both enzymes. Introduction of these alterations into bacterial RNase III permitted a genetic test for domain function: defects were noted upon introduction of residues that form part of the catalytic centre from the second RNase III domain of Dicer family members. Antiparallel alignment of Dicer's RNase III motifs on a dsRNA substrate could produce four compound active sites, but the central two of these would be inactive. In this way, cleavage would

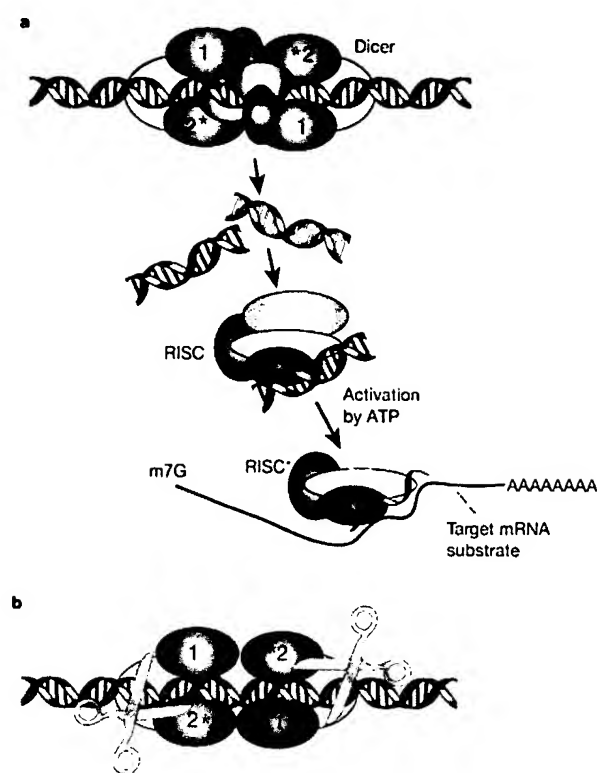


Figure 2 Dicer and RISC (RNA-induced silencing complex). **a**, RNAi is initiated by the Dicer enzyme (two Dicer molecules with five domains each are shown), which processes double-stranded RNA into ~22-nucleotide small interfering RNAs³⁶. Based upon the known mechanisms for the RNase III family of enzymes, Dicer is thought to work as a dimeric enzyme. Cleavage into precisely sized fragments is determined by the fact that one of the active sites in each Dicer protein is defective (indicated by an asterisk), shifting the periodicity of cleavage from ~9–11 nucleotides for bacterial RNase III to ~22 nucleotides for Dicer family members⁴⁰. The siRNAs are incorporated into a multicomponent nuclease, RISC (green). Recent reports suggest that RISC must be activated from a latent form, containing a double-stranded siRNA to an active form, RISC*, by unwinding of siRNAs⁴¹. RISC* then uses the unwound siRNA as a guide to substrate selection³¹. **b**, Diagrammatic representation of Dicer binding and cleaving dsRNA (for clarity, not all the Dicer domains are shown, and the two separate Dicer molecules are coloured differently). Deviations from the consensus RNase III active site in the second RNase III domain inactivate the central catalytic sites, resulting in cleavage at 22-nucleotide intervals.

occur at ~22-base intervals, and subtle alterations in Dicer structure could alter the spacing of these catalytic centres and explain the species-specific variation in siRNA length (A. Denli and G.J.H, unpublished results).

The effector step

In the *Drosophila* system, RNAi is enforced by RISC, a protein–RNA effector nuclease complex that recognizes and destroys target mRNAs. The first subunit of RISC to be identified was the siRNA, which presumably identifies substrates through Watson–Crick base-pairing³¹. Zamore and colleagues have recently shown that RISC is formed in embryo extracts as a precursor complex of ~250K; this becomes activated upon addition of ATP to form a ~100K complex that can cleave substrate mRNAs⁴¹. Cleavage is apparently endonucleolytic, and occurs only in the region homologous to the siRNA. siRNAs are double-stranded duplexes with two-nucleotide 3' overhangs and 5'-phosphate termini^{33,34}, and this configuration is functionally important for incorporation into RISC complexes^{34,41}. However, single-stranded siRNAs should be most effective at seeking

homologous targets, and one intriguing correlation with the transition of RISC zymogens to active enzymes is siRNA unwinding⁴¹.

My laboratory has purified RISC from *Drosophila* S2 cells as a ~500K ribonucleoprotein with slightly different characteristics^{31,42}. In embryo extracts, RISC* (the 100K active RISC species) cleaves its substrates endonucleolytically⁴¹. Intermediate cleavage products are never observed in even the most highly purified RISC preparations from S2 cells, suggesting the presence of an exonuclease in this enzyme complex. Therefore, the complex formed *in vivo* probably contains additional factors that account for observed differences in size and activity. Alternatively, RISC purified from S2 cells may become activated — perhaps changing size and subunit composition — upon incubation with ATP.

RISC from S2 cells co-purifies with AGO2, a member of the *Argonaute* gene family⁴². Argonaute proteins were first identified in *Arabidopsis* mutants that produced altered leaf morphology⁴³, and form a large, evolutionarily conserved gene family with representatives in most eukaryotic genomes, with the possible exception of *Saccharomyces cerevisiae* (reviewed in ref. 44). These proteins are characterized by the presence of two homology regions, the PAZ domain and the Piwi domain, the latter being unique to this group of proteins. The PAZ domain also appears in Dicer proteins, and may be important in the assembly of silencing complexes³⁶.

Argonaute proteins were linked to RNAi by genetic studies in *C. elegans*, whose genome contains >20 related genes. The *rde-1* gene was isolated by Mello and colleagues⁴⁵ from a mutant worm that was unable to sustain RNAi in germline or soma. Using genetic methods, Grishok and colleagues⁴⁵ found a requirement for RDE-1 and RDE-4 for initiation of silencing in a parental animal; however, neither function was required for systemic silencing in F₁ progeny. In contrast MUT-7 (ref. 46) and RDE-2 were both dispensable in the parent, but were required in their progeny.

Rationalizing these results with the simple model proposed above is difficult. Indeed, RDE-4 is a small dsRNA-binding protein, and both RDE-1 and RDE-4 can interact with *C. elegans* Dicer (H. Tabara *et al.*, unpublished data). Perhaps RDE-4 initially recognizes dsRNA and delivers it to the Dicer enzyme. This would be consistent with the observation that siRNA levels are greatly reduced in worms that lack RDE-4 function, but are abundant in worms that lack RDE-1 (ref. 47). Similarly, in *Neurospora*, mutations in the Argonaute family member *qde-2* eliminate quelling (transgene co-suppression), but do not alter accumulation of siRNAs⁴⁸. Thus RDE-1, and perhaps other Argonaute proteins as well, might shuttle siRNAs to appropriate effector complexes (RISCs). Consistent with this notion, we have detected transient interactions in S2 cell extracts between Dicer and Argonaute family members (ref. 42, and A. Caudy, unpublished data). This model has implications for signal amplification and systemic silencing.

Amplification and spreading of silencing

One of the most provocative aspects of RNAi in *C. elegans* is its ability to spread throughout the organism, even when triggered by minute quantities of dsRNA¹. Similar systemic silencing phenomena have been observed in plants, in which silencing could pervade a plant or even be transferred to a naive grafted scion⁴⁹. Accounting for these phenomena requires firstly a system to pass a signal from cell to cell, and secondly a strategy for amplifying the signal.

Recently, a phenomenon termed 'transitive RNAi' has provided some useful clues. Transitive RNAi refers to the movement of the silencing signal along a particular gene (Fig. 3). For example, in *C. elegans*, targeting the 3' portion of a transcript results in suppression of that mRNA and in the production of siRNAs homologous to the targeted region. In addition, siRNAs complementary to regions of the transcript upstream from the area targeted directly by the silencing trigger also appear and accumulate⁵⁰. If these siRNAs are complementary to other RNAs, those are also targeted (hence, 'transitive' RNAi).

In both plants and *C. elegans*, dsRNA-induced silencing requires proteins similar in sequence to a tomato RNA-directed RNA

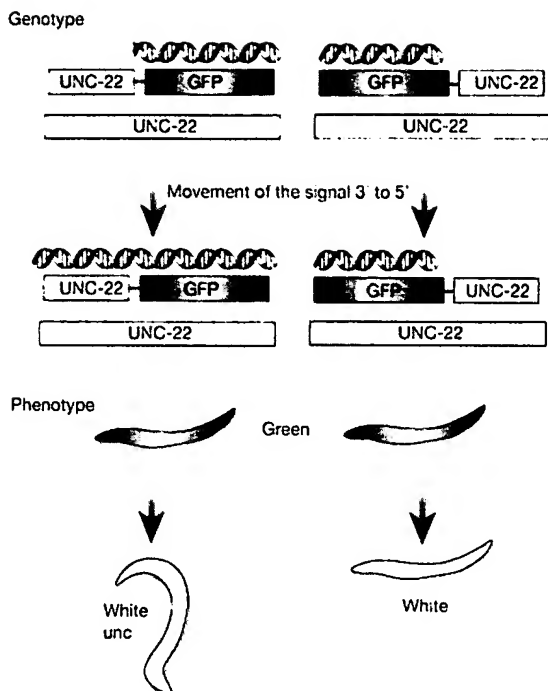


Figure 3 Transitive RNAi. In transitive RNAi in *C. elegans*, silencing can travel in a 3' to 5' direction on a specific mRNA target⁵⁰. The simplest demonstration comes from the creation of fusion transcripts. Consider a fragment of green fluorescent protein (GFP) fused 3' to a segment of UNC-22 (left). Targeting GFP abolishes fluorescence but also creates an unexpected, uncoordinated phenotype. This occurs because of the production of double-stranded RNA and consequently small interfering RNAs homologous to the endogenous UNC-22 gene. In a case in which GFP is fused 5' to the UNC-22 fragment (right), GFP dsRNA still ablates fluorescence but does not produce an uncoordinated phenotype.

polymerase (RdRP)⁵¹, which could be involved in amplifying the RNAi signal. However, only the tomato enzyme has been shown to possess polymerase activity, and biochemical studies will be required to establish definitively the role these proteins play in RNAi. In *Arabidopsis*, SDE1/SGS2 is required for transgene silencing, but not for virally induced gene silencing (VIGS)^{19,52}. This suggests that SDE1/SGS2 may act as an RdRP, as viral replicases could substitute for this function in VIGS. In *Neurospora*, QDE-1 is required for efficient quelling⁵³. EGO-1 is essential for RNAi in the germline of *C. elegans*⁵⁴, and another RdRP homologue, RRF-1/RDE-9, is required for silencing in the soma⁵⁰ (D. Conte and C. Mello, unpublished data).

These genetic studies have led to a model for transitive RNAi in which siRNAs might prime the synthesis of additional dsRNA by RdRPs. RdRP activity has been reported recently from *Drosophila* embryo extracts⁵⁵, although transitive RNAi has yet to be observed in flies. While numerous experiments suggest that an RdRP is not required for RNAi in *Drosophila* extracts, the possibility remains that such an enzyme might act, for example, in triggering RNAi by the production of dsRNA from dispersed, multicopy transgenes.

The fact that RDE-1 and RDE-4 are required only for initiation of RNAi in parental *C. elegans* adds an additional layer of complexity to the model. Perhaps exogenous dsRNAs are recognized initially in a manner that is distinct from recognition of secondary dsRNA, which may be produced by RdRPs. For example, the proposed function of RDE-4 in delivering dsRNA to Dicer could be substituted for secondary dsRNAs by another hypothetical protein. Alternatively, Dicer could exist in a stable complex with an RdRP, making dsRNA delivery unnecessary. The requirement for RRF-1/RDE-9 throughout the

C. elegans soma — and the similar requirement for SDE1/SGS2 in plants — also suggests that most RNAi in these systems is driven by secondary siRNAs produced through the action of RdRPs.

However, other possibilities also exist. Indeed, in plants, transitive RNAi travels in both 3'→5' and 5'→3' directions⁵⁶, which is inconsistent with the simple notion of siRNAs priming dsRNA synthesis. Instead, one can imagine that genomic loci may serve as a reservoir for silencing. In some systems, it is known that exposure to dsRNA can produce alterations in chromatin structure, which could lead to the production of 'aberrant' mRNAs that are substrates for conversion to dsRNA by RdRPs. This model would permit bi-directional spread, as such an expansion of altered chromatin structure is an established phenomenon. Moreover, a similar model could explain co-suppression that is occasionally triggered by single-copy, dispersed transgenes. Finally, this model would be consistent with transitive effects that have been observed for both transcriptional and post-transcriptional silencing in *Drosophila*, which operate in the absence of any homology in the transcribed RNA, and thus differ from 'transitive RNAi' in *C. elegans*^{18,24}. But support for a genome-based amplification model remains elusive, as does the nature of the 'aberrant' RNAs that trigger siRNA formation and an explanation for how chromatin modifications could induce their production.

Although these models suggest mechanisms for cell-autonomous amplification of the silencing signal, the character of the signal that transmits systemic silencing in plants and animals is unknown. Two candidates are siRNAs themselves or long dsRNAs, perhaps formed via RdRP-dependent amplification.

Note that, in plants, two types of transmission must be considered. The first is short-range, cell-to-cell transmission. Plant cells are intimately connected through cytoplasmic bridges known as plasmodesmata. Movement of RNA and proteins via these cell-cell junctions is well known, and it is likely that either long dsRNA or siRNAs could be passed through these connections. But the silencing signal must also be passed over a longer range through the plant vasculature⁵⁷. In this regard, studies of a viral silencing inhibitor have provided evidence against siRNAs being critical for systemic silencing in plants. Hc-Pro suppresses silencing and also interferes with the production of siRNAs from dsRNA triggers⁵⁸. Expression of Hc-Pro does not interfere with transgene methylation, which results in transcriptional gene silencing (TGS) if present in the promoter and which may contribute to post-transcriptional gene silencing (PTGS) if present in the transcribed sequence. Hc-Pro expression in a silenced rootstock relieves silencing and inhibits siRNA production, but a systemic signal can still be passed from this rootstock to an engrafted scion lacking Hc-Pro expression.

Recently, Hunter and colleagues identified a protein in *C. elegans* that is required for systemic silencing⁵⁹. The *sid-1* gene encodes a transmembrane protein that may act as a channel for import of the silencing signal. Expression of *sid-1* is largely lacking from neuronal cells, perhaps explaining initial observations that *C. elegans* neurons were resistant to systemic RNAi. SID-1 homologues are absent from *Drosophila*, consistent with a lack of systemic transmission of silencing in flies, but are present in mammals, raising the possibility that some aspects of RNAi may act non-cell autonomously in mammals.

Other components of the RNAi machinery

A combination of genetics and biochemistry has led to much progress towards understanding the mechanism of PTGS, but many questions remain. In *Drosophila* embryo extracts, pre-RISC becomes activated upon unwinding of siRNAs in an ATP-dependent process. A number of different helicases have been identified in searches for RNAi-deficient mutants (for example, QDE-3, MUT6 and MUT-14), and any of these might be candidates for a RISC activator⁶⁰⁻⁶². Additionally, the identities of RISC-associated nucleases that cleave targeted mRNAs remain elusive. Studies of RISC formed in embryo extracts suggest an endonuclease that cleaves the siRNA-mRNA hybrid near the middle of the duplex, while RISC

formed *in vivo* may have additional exonuclease activities. The MUT-7 protein, which is essential for RNAi in the *C. elegans* germ line, has nuclease homology, but a *Drosophila* relative of this protein has not yet been found in RISC (ref. 46, and S. Hammond, unpublished data). The efficiency of RNAi suggests an active mechanism for searching the transcriptome for homologous substrates. Most *Drosophila* RISC might be associated with the ribosome³¹, and recent studies have extended this observation to trypanosomes (E. Ullu, unpublished data). Finally, relationships between the RNAi machinery and other aspects of RNA metabolism in the cell must be explored. For example, genetic evidence⁶³ suggests a link between RNAi and nonsense-mediated decay, raising the possibility that the RNAi machinery may be important in destruction of improperly processed mRNAs or in the general regulation of mRNA stability.

RNAi and the genome

In plants, dsRNA induces genomic methylation at sites of sequence homology (ref. 22, reviewed in ref. 64). Methylation is asymmetric and is not restricted to CpG or CpXpG sequences. If methylation occurs in the coding sequence, it has no apparent effect on the transcription of the locus, although silencing still occurs at the post-transcriptional level. Methylation of the promoter sequence induces TGS²³, which unlike PTGS is stable and heritable²¹. Thus, dsRNA can clearly trigger alterations at the genomic level, but the degree to which these alterations are relevant to PTGS remains uncertain.

Recent studies have begun to generalize the notion of an intimate connection between the RNAi machinery and the genome, and to draw mechanistic links between PTGS and TGS. For example, in *C. elegans*, *mut-7* and *rde-2* mutations de-repress transgenes that are silenced at the level of transcription by a polycomb-dependent mechanism²⁵. Polycomb-group proteins function by organizing chromatin into 'open' or 'closed' conformations, creating stable and heritable patterns of gene expression. Recently, Goldstein and colleagues found that the polycomb proteins MES-3, MES-4 and MES-6 are required for RNAi, at least under some experimental conditions²⁶. Mutant worms were deficient in the RNAi response if high levels of dsRNA were injected, but were not deficient in the presence of limiting dsRNA. Of course, the effects of these mutants could be indirect, altering the expression of other elements or regulators of the RNAi pathway. However, links between altered chromatin structures and dsRNA-induced gene silencing have also emerged from plant and *Drosophila* systems. In particular, alterations of either methyltransferases (*MET1*) or chromatin remodelling complexes (for example, *DDM1*) can affect both the degree and persistence of silencing in *Arabidopsis*^{21,65}. Conversely, mutations in genes required

for PTGS (for example, *AGO1* and *SGS2*) decrease both co-suppression and transgene methylation⁶⁶. Furthermore, mutation of *piwi*, a relative of the RISC component *Argonaute-2*, compromises co-suppression of dispersed transgenes in *Drosophila* at both the post-transcriptional and transcriptional levels²⁴.

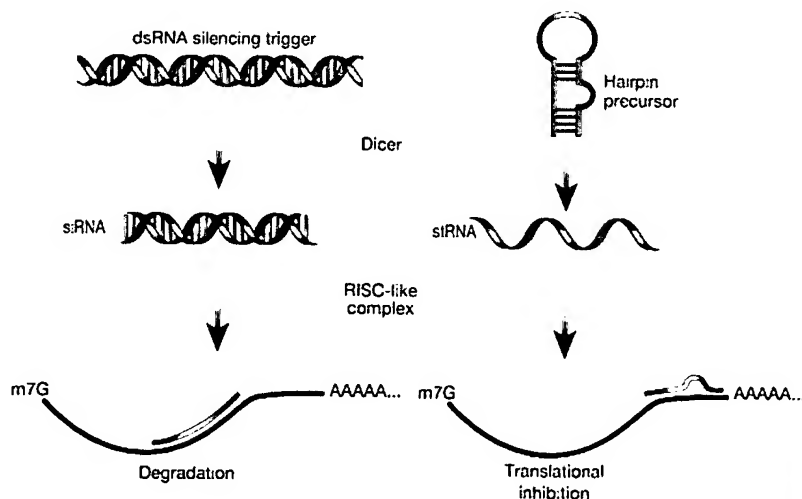
Thus, one of the most fascinating and least-explored responses to dsRNA involves a possible recognition of genomic DNA by derivatives of the silencing trigger, possibly siRNAs. One model suggests that a variant, nuclear RISC carries a chromatin remodelling complex rather than a ribonuclease to its cognate target. Indeed, Martienssen, Grewal and colleagues have recently noted a requirement for relatives of Dicer and RISC components in the silencing of centromeric repeats in *Schizosaccharomyces pombe* (T. Volpe, C. Kidner, I. Hall, S. Grewal and R. Martienssen, personal communication). It seems therefore that a principal biological function of the RNAi machinery may be to form heterochromatic domains in the nucleus that are critical for genome organization and stability.

Biological functions of RNAi

Because target identification depends upon Watson-Crick base-pairing interactions, the RNAi machinery can be both flexible and exquisitely specific. Thus, this regulatory paradigm may have been adapted and adopted for numerous cellular functions. For example, in plants, RNAi forms the basis of VIGS, suggesting an important role in pathogen resistance. An elegant proof of this hypothesis comes from the genetic links between virulence and RNAi pathways (refs 52, 67, and reviewed in ref. 68). Many plant viruses encode suppressors of PTGS that are essential for pathogenesis, and these virulence determinants can be masked by host mutations in silencing pathways. RNAi has also been linked to the control of endogenous parasitic nucleic acids. In *C. elegans*, some RNAi-deficient strains are also 'mutators' owing to increased mobility of endogenous transposons^{25,46}. In many systems, transposons are silenced by their packaging into heterochromatin (reviewed in ref. 64). Therefore, it is tempting to speculate that RNAi may stabilize the genome by sequestering repetitive sequences such as mobile genetic elements, preventing transposition and making repetitive elements unavailable for recombination events that would lead to chromosomal translocations. However, it remains to be determined whether RNAi regulates transposons through effects at the genomic level or by post-transcriptionally targeting mRNAs (for example, those encoding transposases) that are required for transposition.

A role for RNAi pathways in the normal regulation of endogenous protein-coding genes was originally suggested through the analysis of plants and animals containing dysfunctional RNAi components.

Figure 4 Small interfering RNAs versus small temporal RNAs. Double-stranded siRNAs of length ~21–23 nucleotides are produced by Dicer from dsRNA silencing triggers. Characteristic of RNase III products, these have two-nucleotide 3' overhangs and 5'-phosphorylated termini. To trigger target degradation with maximum efficiency, siRNAs must have perfect complementarity to their mRNA target (with the exception of the two terminal nucleotides, which contribute only marginally to recognition). siRNAs, such as *lin-4* and *let-7*, are transcribed from the genome as hairpin precursors. These are also processed by Dicer, but in this case, only one strand accumulates. Notably, neither *lin-4* nor *let-7* show perfect complementarity to their targets. In addition, siRNAs regulate targets at the level of translation rather than RNA degradation. It remains unclear whether the difference in regulatory mode results from a difference in substrate recognition or from incorporation of siRNAs and siRNAs into distinct regulatory complexes.



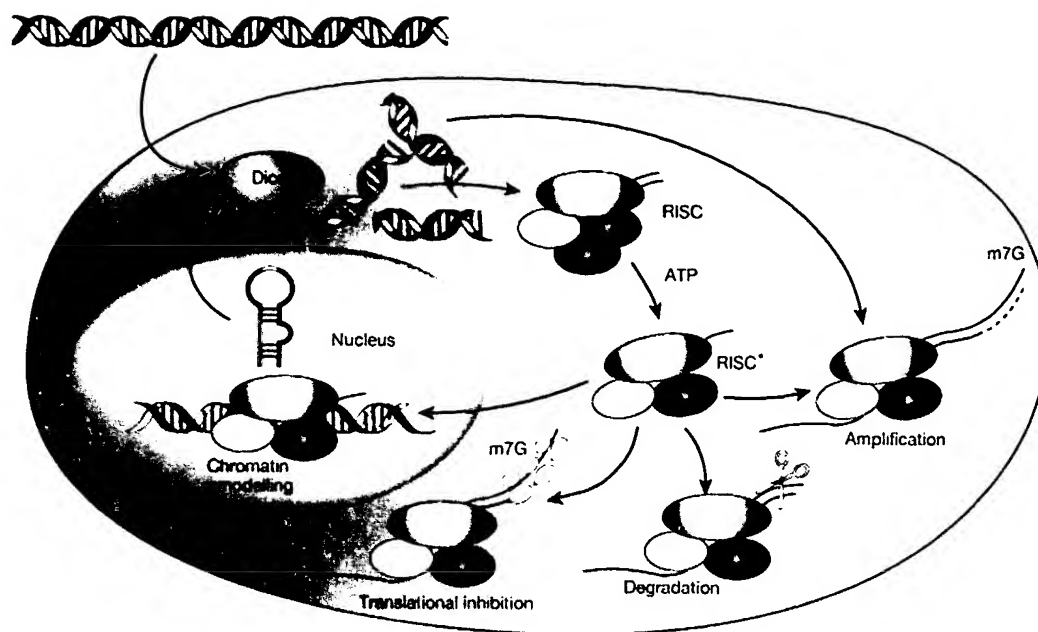


Figure 5 A model for the mechanism of RNAi. Silencing triggers in the form of double-stranded RNA may be presented in the cell as synthetic RNAs, replicating viruses or may be transcribed from nuclear genes. These are recognized and processed into small interfering RNAs by Dicer. The duplex siRNAs are passed to RISC (RNA-induced silencing complex), and the complex becomes activated by unwinding of the duplex. Activated RISC complexes can regulate gene expression at many levels. Almost

certainly, such complexes act by promoting RNA degradation and translational inhibition. However, similar complexes probably also target chromatin remodelling. Amplification of the silencing signal in plants may be accomplished by siRNAs priming RNA-directed RNA polymerase (RdRP)-dependent synthesis of new dsRNA. This could be accomplished by RISC-mediated delivery of an RdRP or by incorporation of the siRNA into a distinct, RdRP-containing complex.

Mutations in the *Argonaute-1* gene of *Arabidopsis*, for example, cause pleiotropic developmental abnormalities that are consistent with alterations in stem-cell fate determination⁴³. A hypomorphic mutation in *Carpel Factory*, an *Arabidopsis* Dicer homologue, causes defects in leaf development and overproliferation of floral meristems⁶⁹. Mutations in *Argonaute* family members in *Drosophila* also impact normal development. In particular, mutations in *Argonaute-1* have drastic effects on neuronal development⁷⁰, and *piwi* mutants have defects in both germline stem-cell proliferation and maintenance⁷¹.

This should not be interpreted as a demonstration that PTGS pathways regulate endogenous gene expression *per se*. In fact, separation-of-function *ago1* mutants have recently been isolated that preferentially affect PTGS⁷² without affecting development. Mutations in *Zwille*, another *Argonaute* family member, also alter stem-cell maintenance⁷³, and this occurs without perceptible impact on dsRNA-mediated silencing⁷². Thus, components of the RNAi machinery, and related gene products, may function in related but separable pathways of gene regulation.

A possible mechanism underlying the regulation of endogenous genes by the RNAi machinery emerged from the study of *C. elegans* containing mutations in their single Dicer gene, *DCR-1*. Unlike most other RNAi-deficient worm mutants, *dcr-1* animals were neither normal nor fertile: the mutation induced a number of phenotypic alterations in addition to its effect on RNAi^{37-39,74}. Intriguingly, Dicer mutants showed alterations in developmental timing similar to those observed in *let-7* and *lin-4* mutants. The *lin-4* gene was originally identified as a mutant that affects larval transitions⁷⁵, and *let-7* was subsequently isolated as a similar heterochronic mutant²⁸. These loci encode small RNAs, which are synthesized as ~70-nucleotide precursors and post-transcriptionally processed to a ~21-nucleotide mature form. Genetic and biochemical studies have indicated that these RNAs are processed by Dicer^{37-39,74}.

The small temporal RNAs (stRNAs) encoded by *let-7* and *lin-4* are negative regulators of specific protein-coding genes, as might be expected if stRNAs trigger RNAi. However, stRNAs do not trigger mRNA degradation, but regulate expression at the translational level^{76,77}. This raised the possibility that stRNAs and RNAi might be linked only by the processing enzyme Dicer. However, Mello and colleagues demonstrated a requirement for Argonaute family proteins (that is, Alg-1 and Alg-2) in both stRNA biogenesis and stRNA-mediated suppression³⁹, which led to a model in which the effector complexes containing siRNAs and stRNAs are closely related, but regulate expression by distinct mechanisms (Fig. 4). Neither LIN-4 nor LET-7 forms a perfect duplex with its cognate target⁷⁸. Thus, in one possible model an analogous RISC complex is formed containing either siRNAs or stRNAs. In the former case, cleavage is dependent upon perfect complementarity, while in the latter, cleavage does not occur, but the complex blocks ribosomal elongation. Alternatively, siRNAs and stRNAs may be discriminated and enter related but distinct complexes that target substrates for degradation or translational regulation, respectively. Consistent with this latter model is the observation that siRNAs or exogenously supplied hairpin RNAs that contain single mismatches with their substrates fail to repress, rather than simply shifting their regulatory mode to translational inhibition^{34,79,80}.

In this scenario, RISC may be viewed as a flexible platform upon which different regulatory modules may be superimposed (Fig. 5). The core complex would be responsible for receiving the small RNA from Dicer and using this as a guide to identify its homologous substrate. Depending upon the signal (for example, its structure and localization), different effector functions could join the core: in RNAi, nucleases would be incorporated into RISC, whereas in stRNA-mediated regulation, translational repressors would join the complex. Transcriptional silencing could be accomplished by the inclusion of chromatin remodelling factors, and one could imagine other adaptations might exist.

Whether or not RISC is a flexible regulator becomes particularly important in light of recent findings that *let-7* and *lin-4* are archetypes of a large class of endogenously encoded small RNAs. Over 100 of these microRNAs or miRNAs have now been identified in *Drosophila*, *C. elegans* and mammals^{81–84}, and although their functions are unknown, their prevalence hints that RNAi-related mechanisms may have pervasive roles in controlling gene expression. In this regard, a number of miRNAs from *Drosophila* are partially complementary to two sequences, the K box and the Brd box, that mediate post-transcriptional regulation of numerous mRNAs⁸⁵.

RNAi and genomics

RNAi has evolved into a powerful tool for probing gene function. In *C. elegans*, testing the functions of individual genes by RNAi has now extended to analysis of nearly all of the worm's predicted ~19,000 genes (J. Ahringer, unpublished data). Similar strategies are being pursued in other organisms, including plants (D. Baulcombe and P. Waterhouse, personal communication). Although it seemed for some time that deploying RNAi in mammalian systems would not be feasible, the first hint that the technology might work came when RNAi was demonstrated in early mouse embryos^{86,87}. But this appeared to be of limited utility, as mammalian somatic cells, but not some embryonic cells, exhibit nonspecific responses to dsRNA which would obscure sequence-specific silencing. One of these is the RNA-dependent protein kinase (PKR) pathway, which responds to dsRNA by phosphorylating EIF-2 α and nonspecifically arresting translation⁸⁸. Tuschl and colleagues then showed that siRNAs themselves could be used to induce effective silencing in many mammalian cells⁷⁹. These small RNAs, which are chemically synthesized mimics of Dicer products, are presumably incorporated into RISC and target cognate substrates for degradation. The siRNAs are too small to induce nonspecific dsRNA responses such as PKR⁸⁹.

One drawback that siRNAs have is that their effects are transient, as mammals apparently lack the mechanisms that amplify silencing in worms and plants. In several systems, including plants, *Drosophila*, *C. elegans* and trypanosomes, RNAi has been made stable and heritable by enforced expression of the silencing trigger, usually as an inverted repeat sequence forming a hairpin structure *in vivo*^{90–95}. We have reported mammalian cell lines in which genes are stably suppressed by RNAi through the expression of a 500-base-pair dsRNA⁹⁶. However, this approach was limited to cell types that lacked generic responses to dsRNA such as the PKR pathway. Recently, we and others have shown that short hairpin RNAs (shRNAs) modelled on miRNAs can be used to manipulate gene expression experimentally^{80,97,98}. These may be expressed *in vivo* from RNA polymerase III (Pol III) promoters to induce stable suppression in mammalian cells.

The availability of stable triggers of RNAi builds upon the utility of siRNAs in several ways. Induced phenotypes can now be observed over long time spans. Stably engineered cells can be assayed either *in vitro* or *in vivo*, perhaps testing the angiogenic or metastatic potential of tumour cells in xenograft models. RNAi may potentially be used to create hypomorphic alleles rapidly in transgenic mice. If inducible Pol III promoters were used^{99,100}, this could permit a powerful approach akin to the use of tissue-specific Gal4-drivers in *Drosophila*. Finally, shRNAs could be combined with existing high-efficiency gene delivery vehicles to create *bona fide* RNAi-based therapeutics. In this regard, we have successfully delivered shRNAs from replication-deficient retroviruses, and foresee numerous applications for *ex vivo* manipulation of stem cells based upon this paradigm. For example, a patient's own bone marrow stem cells could be engineered to resist HIV infection by targeting either the HIV RNA itself or receptors necessary for HIV infection (for example, CCR5). Furthermore, we see no conceptual barrier to incorporating this strategy for targeted suppression into adenovirus or herpesvirus-based delivery vehicles. Ultimately, the exquisite specificity of RNAi may make it possible

to silence a disease-causing mutant allele specifically, such as an activated oncogene, without affecting the normal allele.

Perspective

Over the past few years, the way in which cells respond to dsRNA by silencing homologous genes has revealed a new regulatory paradigm in biology. This response can be triggered in many different ways, ranging from experimental introduction of synthetic silencing triggers to the transcription of endogenous RNAs that regulate gene expression. We are only beginning to appreciate the mechanistic complexity of this process and its biological ramifications. RNAi has already begun to revolutionize experimental biology in organisms ranging from unicellular protozoans to mammals. RNAi has been applied on the whole-genome scale in *C. elegans* and this goal is being pursued in plant systems. My laboratory, as part of the larger cancer genomics effort, has undertaken to target, individually, every gene in the human genome using expressed shRNAs. This will permit large-scale loss-of-function genetic screens and rapid tests for genetic interactions to be performed for the first time in mammalian cells. Such approaches hold tremendous promise for unleashing the dormant potential of sequenced genomes. □

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Specific interference with gene expression and gene function mediated by long dsRNA in neural cells

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Abstract

Double-stranded (ds) RNA-induced sequence-specific interference with gene expression, RNA interference (RNAi), has been extensively used in invertebrates, allowing for efficient and high-throughput gene silencing and gene function analysis. In vertebrates, however, use of RNAi to study gene function has been limited due to non-specific effects induced by double-stranded RNA (dsRNA)-dependent protein kinase and interferon activation. dsRNA-induced specific inhibition of vertebrate gene expression has only been shown in embryonic and non-differentiated mammalian cells. In this report, we demonstrate dsRNA-induced specific interference of gene expression and gene function in partially as well as fully differentiated mouse neuroblastoma cells. Specific silencing was observed in the expression of an integrated transgene coding for green fluorescent protein and a variety of endogenous genes. Moreover, we show that RNAi-mediated inhibition of poly (ADP-ribose) polymerase (PARP) expression induced cellular resistance to oxygen–glucose deprivation, consistent with the role of PARP in ischemia-induced brain damage. Our results indicate that RNAi can be used as a powerful tool to study gene function in neural cells.

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Keywords: RNAi; Neural cells; Differentiation; Gene-silencing; Validation; Oxygen–glucose deprivation

1. Introduction

Specific gene silencing mediated by double-stranded RNA (dsRNA), or RNA interference (RNAi), has been widely used to study gene function in diverse invertebrate systems, such as *C. elegans* and *Drosophila* (Fire et al., 1998; Kennerdell and Carthew, 1998; Hammond et al., 2000; Sharp and Zamore, 2000). The identification of a small RNA species of ~25 bp nucleotides in plants undergoing post-transcriptional silencing (PTGS) provided a key insight into the understanding of the mechanistic basis of RNAi (Hamilton and Baulcombe, 1999). In cell-free systems with extracts of *Drosophila* embryos and Schneider cells, dsRNA is processed into 21–25 nt small inhibitory RNA (siRNA), which guides the degradation of target mRNA at 21–23 nt intervals (Tuschl et al., 1999; Zamore et al., 2000).

Unlike invertebrate cells, most mammalian cells respond to dsRNAs longer than 30 bp by inducing interferon α and β expression and activating dsRNA-dependent protein kinase (PKR) and 2', 5' oligoadenylate synthetase, leading to a general inhibition of protein synthesis and non-specific degradation of mRNA by RNase L (Stark et al., 1998). However, potent and specific dsRNA-mediated inhibition of gene expression was observed in mouse embryos microinjected with long dsRNA, suggesting the existence of the RNAi machinery in mammalian cells (Wianny and Zernicka-Goetz, 2000). Use of RNAi in mammalian cells was further validated and greatly expanded by Tuschl and colleagues, in which siRNAs were used to induce gene-specific silencing in a variety of mammalian cell lines (Elbashir et al., 2001). However, potent and specific RNAi-mediated silencing was not achieved with long dsRNA in commonly used mammalian cells, including HeLa, HEK293, CHO, NIH3T3 cells, due to non-specific inhibition of gene expression (Ui-Tei et al., 2000; Elbashir et al., 2001). Recent studies reported that long dsRNA-induced efficient RNAi in non-differen-

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tiated mouse embryonic teratocarcinoma cell lines which are deficient in some of the dsRNA- and IFN-activated enzymes (Billy et al., 2001; Yang et al., 2001; Paddison et al., 2002).

In this report we show that dsRNAs with an average size of 500–700 base pairs are able to mediate gene-specific silencing of an integrated transgene and endogenous genes in partially and fully differentiated mouse neuroblastoma AGYNB010 cells, derived from N2a cells. Using AGYNB010 cells that stably express green fluorescent protein (GFP), we show that the expression level of GFP is significantly attenuated by dsRNA derived from the GFP ORF, but not by dsRNA derived from unrelated genes. In addition, we have shown efficient silencing of several endogenous genes involved in apoptosis, housekeeping, and signal transduction. RNAi-mediated gene silencing was then used as an efficient knockdown tool to confirm the role of an endogenous gene, PARP, in oxygen–glucose deprivation (OGD)-induced neuronal damage (Eliasson et al., 1997). Transfection of dsRNA derived from either the N-terminal or C-terminal part of PARP ORF strongly inhibited expression of PARP. Moreover, inhibition of PARP expression induced cellular resistance to OGD, which is consistent with the role of the PARP protein in ischemia-induced brain damage. Our results indicate that RNAi-mediated gene silencing is a useful tool to elucidate gene function in these neural cells.

2. Methods

2.1. Double-stranded RNA

Sequence-specific primers were used to generate partial clones to be inserted into PCR4TOPO, which served as templates for *in vitro* transcription to generate sense and antisense transcripts using T3 and T7 polymerases (Ambion). An equal molar ratio of sense and antisense transcripts were heated at 60 °C for 30 min and cooled to room temperature for more than 15 min for annealing. The dsRNA preparation was treated with RNase T1 to eliminate any remaining single-stranded RNA. The quality of the dsRNA preparations was analyzed on 1.2% native agarose gels. Primers used for PCR were:

Fragment corresponding to the N-terminal fragment of PARP, PARP-N: AGAAGGATGGCG-GAGGCCTCGG and CTGCTTTCTGGAGG-GAATATTC;

Fragment corresponding to the C-terminal fragment of PARP, PARP-C: AGGCCGCTACTC-

TATCCTCAGCGA and CTCCTGA-GATGTGTGGCAGTAGT.

For other endogenous genes selected to be silenced, the locations of the primers are listed in Fig. 2B.

2.2. Culture and transfection of cells

A cell population, AGYNB010, was derived from N2a cells (ATCC, CCL-131) by growing in growth medium containing Dulbecco's modified minimal essential medium (DMEM, high glucose) (Invitrogen) for more than 30 generations. AGYNB010 cells show higher sensitivity to OGD than N2a cells (data not shown). The growth medium was supplemented with 10% (v/v) fetal bovine serum (Invitrogen), penicillin (100 units/ml) and streptomycin (100 µg/ml). For differentiation, AGYNB010 cells were cultured in Neurobasal medium (Invitrogen) supplemented with N2 supplement (Invitrogen) and 20 µM *all-trans* retinoic acid (Sigma). The NB010-GFP stable cell line was generated by cotransfecting AGYNB010 cells with a GFP expression plasmid and pCMV-script (Stratagene). Cells resistant to G418 were replated at lower density to isolate independent clonal lines for further experiments. dsRNA was transfected in AGYNB010 or NB010-GFP cells using lipofectamine per manufacturer's instructions. Cells were harvested for total RNA or protein extraction one to four days after transfection.

2.3. Western blot and RT-PCR and real time PCR

Protein concentration was determined using the BCA system (Pierce). Anti-GFP (Chemicon International Inc., CA) or anti-PARP antibodies (Oncogene, CA) were used to detect the GFP or PARP protein. An equal amount of protein (30 µg) was loaded in each lane. The Western blot was then scanned and quantified using NIH image (W. Rasband, NIH). For reverse transcription, the same amount of RNA (0.3–5 µg) was used for reverse-transcription with SuperscriptII (Invitrogen) and a modified oligo-dT primer in the presence of Superase-In RNase inhibitor (Ambion). SYBR Green real-time PCR amplifications were performed in an iCycler Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA) to estimate changes in gene expression after transfection with dsRNA. Primers (Operon Technologies, Alameda, CA) were designed using Primer3 (Whitehead Institute for Biomedical Research), the locations of which are listed in Fig. 2B. The standard curves were used to calculate the PCR efficiency of the primer set. GAPDH was used as internal control. All PCR reactions were performed in duplicate or triplicate. Quantification was performed using the comparative cycle threshold (CT) method, where CT is defined as the cycle number at which

fluorescence reaches a set threshold value. The target transcript was normalized to an endogenous reference (simultaneous triplicate GAPDH reactions), and relative differences were calculated using the PCR efficiency.

2.4. *In vitro* cell proliferation assay

Approximately 1×10^4 AGYNB010 cells per well were plated in a 96 well plate and maintained in either regular growth medium or differentiation medium containing retinoic acid for 1–3 days. Cell proliferation was quantified based on the measurement of BrdU incorporation during DNA synthesis using the Cell Proliferation ELISA (Roche). BrdU was added to the culture medium approximately 18 h before the assay. Cells were fixed with 4% formaldehyde, quenched and permeabilized before anti-BrdU-POD was added. Subsequent assays were conducted after the addition of substrate. The amount of BrdU incorporation was determined using a microplate luminometer LB96V (EG&G Berthold).

2.5. Oxygen–glucose deprivation and cell death quantification

dsRNAs corresponding to the N-terminal fragment of the PARP gene or dsRNA-GFP were transfected into AGYNB010 cells. Four days later, OGD was performed by a complete exchange of normal growth media with deoxygenated, glucose-free Earle's balanced salt solution (EBSS) containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 1 mM NaH₂PO₄, and 0.9 mM CaCl₂, bubbled with 5% H₂/85% N₂/5% CO₂. The cultures were kept in an anaerobic chamber for 3–6 h containing the gas mixture, 5% H₂/85% N₂/5% CO₂, and maintained at 37 °C. OGD was terminated by removal of the cultures from the chamber and replacement of the EBSS solution with oxygenated growth media. OGD-induced cell death was quantified 18–24 h after the termination of OGD using the LIVE/DEAD viability staining assay (Molecular Probes) and quantified on a cytofluor multi-well plate reader (Series 4000, PerSeptive Biosystems).

3. Results

3.1. Inhibition of GFP expression in AGYNB010 cells stably expressing GFP

To demonstrate whether long dsRNA mediates gene-specific silencing in mouse neuroblastoma AGYNB010 cells, we first chose the transgene GFP as a reporter gene. Unlike other studies in which the expression plasmid coding for the reporter gene was cotransfected with dsRNA in a transient manner, we generated an NB010-GFP cell line that expresses GFP protein stably

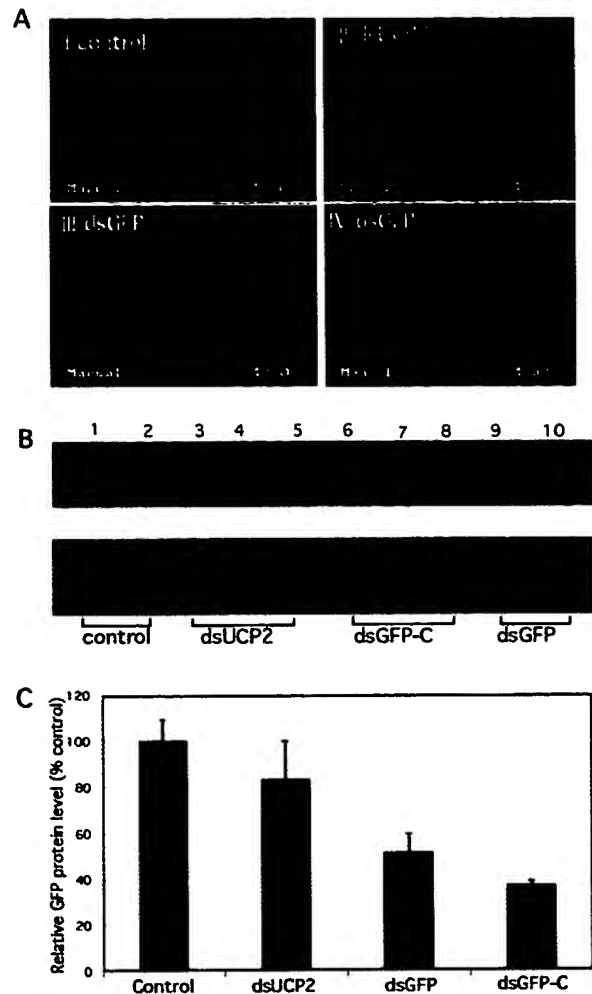


Fig. 1. Effects of dsGFP RNA on the expression of GFP in AGYNB010 cells. (A) Expression of GFP was evaluated in NB010-GFP stable cells transfected with lipofectamine alone (Panel I), 5 nM dsUCP2 RNA (Panel II), 5 nM dsGFP (Panel III) and 8 nM dsGFP (Panel IV). The exposure time for each fluorescent micrograph was 1/30 s as indicated. (B) Western blot shows protein levels of GFP (top) and MAP2 (bottom) in cells transfected with lipofectamine alone (lanes 1–2), 5 nM dsRNA-UCP2 (lanes 3–5), 5 nM dsGFP-C (lanes 6–8), and 5 nM dsGFP (lanes 9–10). (C) Quantification of GFP protein levels from Western blots. Values shown are means \pm SD obtained from three independent experiments.

to mimic the expression of an endogenous gene. dsRNA corresponding to the full-length (dsGFP) or to the C-terminal part of the GFP (dsGFP-C) gene was generated and transfected into these NB010-GFP cells. Control dsRNA corresponding to the entire coding region of uncoupling protein-2 (dsUCP2) was also tested. As shown in Fig. 1A, green fluorescence decreased significantly in cells transfected with dsGFP (panels III and IV) compared with cells transfected with dsUCP-2 (panel II) and mock transfected controls (panel I). Western blot analysis confirmed that dsGFP and dsGFP-C RNA decreased the GFP protein level significantly ($P < 0.05$) without affecting the expression of the housekeeping gene MAP-2 (Fig. 1B and C). In

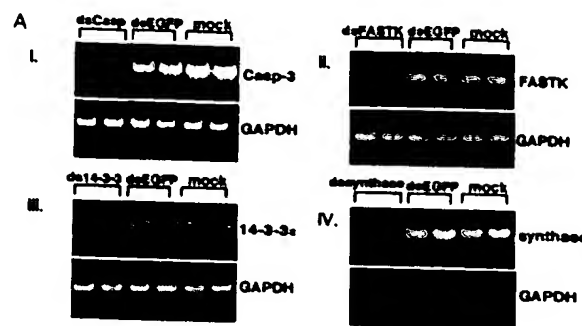
contrast to what was observed in other types of mammalian cells (Elbashir et al., 2001), the slight suppression of GFP expression induced by control dsRNA (dsUCP2) was not statistically significant ($P > 0.05$, Fig. 1C). In addition, dsGFP RNA mediated a decrease in GFP protein in a concentration-dependent manner (data not shown). Our results strongly indicate that long dsRNA mediates gene-specific silencing of an integrated transgene in neuroblastoma cells without inducing significant non-specific mRNA degradation and/or suppression of protein synthesis.

3.2. Inhibition of endogenous gene expression using dsRNA

To test for RNAi-mediated gene silencing of endogenous genes we chose several types of genes, including genes involved in apoptotic pathways such as caspase-3, p53, and 14-3-3 ϵ , signaling pathways such as MAP kinase p38, fas-activated serine threonine kinase (FASTK), and housekeeping enzymes such as 3-hydroxy-3-methylglutaryl coenzyme A synthase 1. To quantify changes in gene expression after dsRNA transfection, we conducted RT-PCR and real time PCR experiments using an iCycler Real-Time detection system (Bio-Rad Laboratories, Hercules, CA).

The dsRNAs corresponding to the above mentioned mouse genes have an average length of 600–800 bps. Fig. 2A shows dsRNA-induced gene-specific silencing of 14-3-3 ϵ , caspase-3, synthase 1, and FASTK without affecting the GAPDH levels. Moreover, little or no non-specific silencing effect was induced by control dsGFP RNA. Fig. 2B describes the names of the selected genes, their NCBI accession numbers, the regions from which dsRNAs were derived, the positions of the primers used for real-time PCR and the percent knockdown achieved. Real-time PCR confirmed that the efficiency of inhibition ranged from 65% to 98%, depending on specific genes. These results further indicate that the RNAi machinery is highly active in neuroblastoma cells and that the silencing effects we observed at the protein level are mediated transcriptionally.

All the effective dsRNAs corresponded to the full-length or portions of the ORF and shared 100% sequence identity with endogenous sequences. We then tested whether effective dsRNA can be derived from a non-ORF region and whether 100% sequence identity is required for efficient silencing of the cognate message. Efficient silencing was observed in cells transfected with dsRNA corresponding to the partial ORF of the mouse p38 gene (Fig. 2B). Interestingly, dsRNA corresponding to the 3'UTR of the rat p38 gene (dsRNA-rat-p38), which only shares about 80% identity with the mouse ortholog in that region, also induced efficient silencing of mouse p38 mRNA in neuroblastoma cells (Fig. 2B). This result indicates that effective dsRNA may not be



Gene name ¹	Accession Number ²	dsRNA Region ³	primer location ⁴ (real-time PCR)	% of knockdown ⁵
mCaspase3	NM_009810	2-723	675, 767	87.3±4.34
mFAST	NM_023229	67-640	984, 1113	65.8±7.34
mp53	M13873	102-881	714, 839	65.7±7.68
mCoenzyme A synthase 1	BC013443	174-583	1304, 1422	73.7±9.17
m14-3-3 ϵ	D87663	284-774	575, 692	98.7±0.41
mp38	NM_011951	321-828	579, 713	89.7±4.34
mp38	NM_031020.1*	1606-2204	579, 713	85.0±1.91

Note: ¹Name of the genes selected for dsRNA silencing.

²NCBI accession numbers for the sequences from which the dsRNAs were derived

³Locations of the partial clones in mRNA from which dsRNA were derived

⁴Starting positions of the primers used for real time PCR: forward and reverse.

⁵% of knockdown is calculated as

$$\left(1 - \frac{\text{Expression level in cells transfected with dsRNA}}{\text{Expression level in cells transfected with control dsRNA (dsGFP)}} \right) \times 100$$

*Efficient inhibition of mouse p38 expression was observed using the dsRNA derived from the 3' UTR of rat p38, which shares about 80% homology with the mouse sequence.

Fig. 2. Inhibition of expression of endogenous genes quantified by RT-PCR. (A) RT-PCR shows dsRNA-induced inhibition of expression of caspase-3, fas-activated kinase (FASTK), 14-3-3 ϵ and 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 induced by dsRNA. Levels of GAPDH expression served as internal controls for the quality and quantity of cDNAs. Equal amounts of cDNA were used in each reaction. (B) Table shows dsRNA-mediated inhibition of expression of endogenous genes, quantified with real-time PCR. Quantification was performed using the comparative CT method. The target transcript was normalized to an endogenous reference (simultaneous triplicate GAPDH reactions), and relative differences were calculated using the PCR efficiency. Data represented were mean \pm SD obtained from two independent experiments performed in duplicates.

restricted to sequences in the ORF, and that 100% sequence identity is likely not required in some cases.

Recent studies show that long dsRNA-induced gene-specific silencing in non-differentiated and embryonic cells (Billy et al., 2001; Yang et al., 2001; Paddison et al., 2002). In our experiments, AGYNB010 cells undergo serum withdrawal during the transfection procedure, which induces partial differentiation (L. Gan and S. Ye, unpublished observation). We then tested whether RNAi is active in fully differentiated AGYNB010 cells. Cells were transfected with 5 nM ds14-3-3 ϵ RNA and then grown in Neurobasal medium in the presence of 20 μ M retinoic acid to induce neuronal differentiation (Mao et al., 2000). After 3 days, AGYNB010 cells developed long processes and cell proliferation was largely inhibited as measured by BrdU incorporation (Fig. 3A–B). In these fully differentiated neural cells,

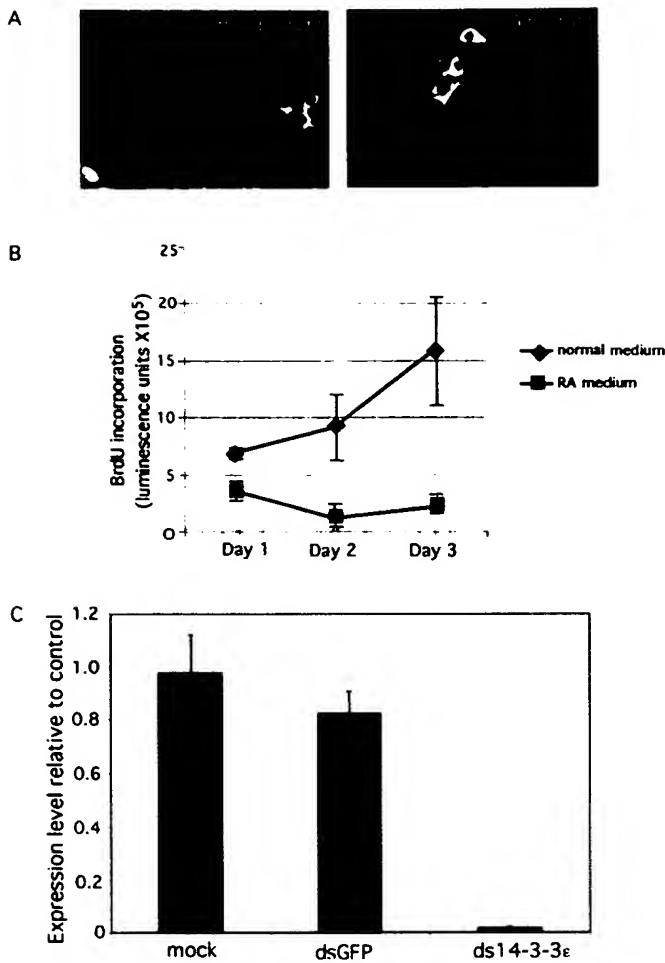


Fig. 3. Effect of dsRNA in differentiated AGYNB010 cells. (A) Cells incubated in differentiation media containing retinoic acid show much longer processes compared with cells incubated in normal media. Cells were immunostained with an anti-tubulin antibody and visualized under a fluorescent microscope. (B) BrdU incorporation was measured in cells incubated in differentiation media and normal media for 1, 2 and 3 days. Data presented were means \pm SD from four independent samples. (C) Real-time PCR was used to measure the levels of 14-3-3ε mRNA from cells transfected with lipofectamine alone, ds14-3-3ε and dsGFP RNAs. Data represented are relative expression levels normalized to cells transfected with dsGFP. Values shown are means \pm SD from three independent experiments performed in duplicates.

ds14-3-3ε induced almost complete inhibition of 14-3-3ε expression ($97.8\% \pm 1.3$), while control dsGFP induced no significant inhibition (Fig. 3C). These results indicate that RNAi is not restricted to non-differentiated cells or cells of embryonic origin, but also active in these fully-differentiated neural cells.

3.3. Inhibition of PARP expression and validation of PARP function using RNAi

One of the most powerful tools to determine the function of a gene is to silence its expression. We have demonstrated that mouse neuroblastoma AGYNB010 cells show a very robust RNAi effect, suggesting that

RNAi can be used for studying gene function in these cells. Hence, we tested the effect of RNAi-induced inhibition of PARP in ischemia-induced cellular damage. PARP was chosen because of its well-established role in ischemia-induced brain damage, since PARP knockout mice are resistant to global cerebral ischemia (Eliasson et al., 1997). As an *in vitro* model for ischemic damage, AGYNB010 cells were exposed to OGD for 3–6 h, and cell viability was assessed 24 h later.

The dsRNAs corresponding to the N-terminal (dsPARP-N) and C-terminal (dsPARP-C) part of the PARP coding region were generated and transfected into AGYNB010 cells. dsGFP was used as control. Four days after transfection, cell lysates were collected and PARP expression was assessed by Western blot analysis (Fig. 4A). Compared with cells transfected with

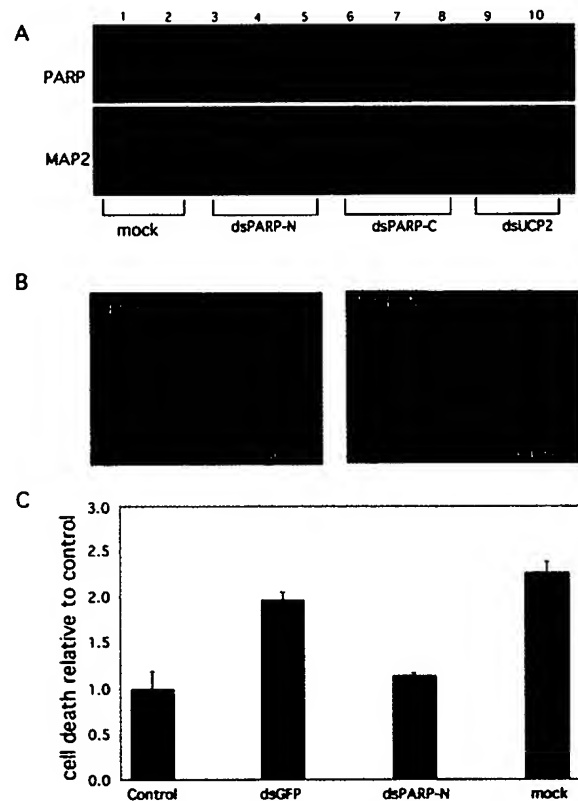


Fig. 4. Inhibition of endogenous PARP gene by dsPARP RNA and subsequent effects on OGD in AGYNB010 cells. (A) Western blot shows PARP expression in cells transfected with 5 nM dsPARP-N (lanes 3–5), 5 nM dsPARP-C (lanes 6–8), and 5 nM of dsUCP2 (lanes 9–10) 4 days after transfection. Each lane represents separate transfections performed within the experiment. (B) The AGYNB010 cells transfected with 5 nM of dsGFP as control or 5 nM of dsPARP-N were challenged with 6 h OGD 4 days after transfection. Cells were stained with calcein AM (green) and EthD-1 (red) (Molecular Probes) and visualized under a Zeiss fluorescent microscope. (C) Cell death was quantified using a multi-well cytofluor plate reader for EthD-1 (excitation wavelength at 530 nm). Values shown are relative cell death after OGD compared with the non-OGD mock-transfected samples. Results presented are means \pm SD from three independent samples. Similar results were obtained in two independent experiments.

control dsGFP, dsPARP-N and dsPARP-C induced a dramatic reduction of PARP protein levels, and dsPARP-N appeared to be more effective than dsPARP-C (Fig. 4A). Four days after transfection with dsPARP-N and dsGFP, cells were exposed to OGD for 6 h. After 18–24 h recovery, cell survival was assessed using double staining with Ethidium homodimer-1 (EthD-1) and calcein AM (Molecular Probes), (Fig. 4B) and a multi-well fluorescence plate reader (Cytofluor, Perseptive System), (Fig. 4C). Cells transfected with dsPARP-N showed significant protection against OGD-induced cell death compared with mock-transfected cells and those transfected with dsGFP. This result confirms previous reports on the essential role of PARP as a mediator in ischemia-induced neuronal damage and, most importantly, validates the use of RNAi in the analysis of the potential role of novel and known genes in ischemia-induced neuronal damage.

4. Discussion

In this report, we show for the first time long dsRNA-induced gene-specific silencing in mouse neuroblastoma AGYNB010 cells under fully or partially differentiated conditions. We demonstrate dsRNA-induced gene-specific silencing of an integrated transgene as well as of a variety of endogenous genes involved in apoptosis, signaling, and housekeeping functions. Control dsRNA did not induce non-specific inhibition of the cognate gene expression. Expression of unrelated housekeeping genes, MAP2 and GAPDH, was not affected by the transfection of dsRNA. Moreover, we show that inhibition of PARP expression with dsRNA corresponding to portions of the PARP ORF offers resistance to OGD. These studies suggest that the RNAi approach may provide an efficient way to knock down gene expression and study gene function in this neural cell system.

The profound gene-specific silencing induced by dsRNA was first discovered in *C. elegans*; and has been used widely as an invaluable tool for studying gene function in a variety of invertebrate species (Clemens et al., 2000; Fraser et al., 2000). However, efforts to use long dsRNA for gene-specific silencing and functional validation in vertebrates have been hindered by the observation that long dsRNA (> 30 bp) induces non-specific inhibition of gene expression. It is well known that in most mammalian cells dsRNA induces an interferon response and initiates a signaling pathway, which includes activation of 2,5-polyadenosine synthetase, RNase L and RNA-dependent kinase PKR (Minks et al., 1979; Manche et al., 1992). These processes finally lead to non-specific inhibition of protein synthesis, and non-specific degradation of both dsRNA and endogenous mRNA. Indeed, a 10- to 20-fold non-specific

silencing by long dsRNA was observed in several mammalian cell lines, masking the modest 2- to 5-fold sequence-specific silencing (Elbashir et al., 2001). Several recent reports showed that long dsRNA-induced gene-specific silencing in PKR-deficient mammalian cells, including embryonic carcinoma P19 and F9 cells as well as mouse embryonic stem (ES) cells (Billy et al., 2001; Yang et al., 2001; Paddison et al., 2002).

We demonstrate that RNAi is highly active in a neuroblastoma AGYNB010 cell line derived from N2a cells, in which long dsRNA induces gene-specific silencing without inducing non-specific inhibition of protein synthesis and gene transcription. In contrast to the undifferentiated P16, F9 and ES cells, the neuroblastoma cells used in our study were either partially differentiated through serum withdrawal or fully differentiated by retinoic acid, suggesting that the functional RNAi machinery is not restricted to non-differentiated cells or cells of embryonic origin in mammals. Whether differentiated and non-differentiated cells utilize similar mechanisms in RNAi-induced silencing warrants further investigation.

While it is well known that P16, F9, and ES cells are deficient in dsRNA-induced IFN response, AGYNB010 cells show no apparent deficiency in such response. Indeed, transfection of long dsRNA (700 bps) derived from the FASTK ORF into AGYNB010 cells induced expression of IFN- β , while transfection of siRNA did not (L. Gan and K.E. Anton, unpublished observation). However, the dsRNA-induced IFN response did not lead to significant non-specific blockade of protein synthesis or degradation of mRNA in these neural cells. We are currently investigating whether upregulation of IFN- β fails to activate PKR, RNase L and other antiviral signaling pathways in these cells. Studies have shown that siRNA of 21 nt can induce efficient gene-specific silencing in mammalian cells (Elbashir et al., 2001). The siRNA derived from the FASTK ORF also induced gene-specific silencing in AGYNB010 cells (Data not shown). We are currently investigating whether long dsRNA is processed into 21–23 nt siRNA to induce degradation of target mRNA in these neural cells.

There is no apparent requirement on the location of dsRNA to induce RNAi and successful knockdown was achieved using dsRNA corresponding to a part of an ORF or 3'UTR, underlining the potential of RNAi as a valuable alternative to antisense technology for gene silencing. Interestingly, different genes require different post-transfection times for efficient inhibition of gene expression. For example, dsp53 RNA induced maximal inhibition in 24–48 h, while both dsPARP and dsGFP induce maximal inhibition in 96 h. We speculate that mRNA and protein stability and turnover time influences the time required to observe a RNAi effect. In this

example, GFP is much more stable than the p53 protein, which has a half-life of 20 min (Soussi, 2000).

The demonstration that dsRNA in a mouse neuroblastoma cell line lacks the non-specific effects present in other mammalian cell lines suggests that these cells can be used to test the specific function of selected genes in cellular responses to diverse stimuli. As an example, we show that dsRNA-mediated inhibition of PARP expression results in a resistance to OGD in mouse neuroblastoma cells. Thus, specific gene silencing induced by dsRNA can be a powerful tool in the analysis of gene function in these neural cells after a pathological challenge.

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Neuron-specific regulation of major histocompatibility complex class I, interferon- β , and anti-viral state genes

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Abstract

The regulation of major histocompatibility complex (MHC) class I, interferon (IFN)- β , and anti-viral state expression in neurons was analyzed. Treatment of neurons with either double-stranded RNA (poly I:poly C) or virus, but not IFNs, induced high levels of IFN- β , but not MHC class I genes. However, neurons treated with IFN- β established an anti-viral state. Transfection of neurons with IFN- β constructs showed that a region containing PRDI (IRF-E site) and PRDII (κ B site) mediated induction, but closely related sites in a MHC class I construct did not. Gel mobility shift assays indicated that transcription factors containing the RelA (p65) component of NF- κ B, but not p50, bound to PRDII. PRDI, however, bound to transcriptional antagonist IRF-2. Unique selective induction of these transcription factors is likely to mediate non-coordinate expression of IFN- β , MHC class I, and anti-viral state genes in neurons.

Keywords: Neurons; Interferon- β ; Major histocompatibility complex class I; Virus; Astrocytes

1. Introduction

The central nervous system (CNS) is involved in the regulation of various functions critical to adaptive responses to environmental stimuli. Neurons, relative to other CNS cells, are most essential to CNS function and have limited regenerative capacity. Therefore, damage to neurons often leads to severe clinical deficits that are generally irreversible. For this reason, structures and mechanisms have evolved to protect these cells from physical, chemical, and immune-mediated damage, including glial cell functions, the blood–brain barrier, and CNS immunoprivilege.

Recent attention has focussed on mechanisms of CNS immunoprivilege (Massa, 1989; Cserr and Knopf, 1992; Streilein, 1993) and specific sparing of virus-infected neurons from major histocompatibility complex

(MHC) class I-restricted cytotoxic anti-viral lymphocytes (Oldstone et al., 1986; Joly et al., 1991; Levine et al., 1991; Sedgwick and Dorries, 1991; Griffin et al., 1992; Joly and Oldstone, 1992; Mucke and Oldstone, 1992). There is evidence that neurons infected with neurotropic viruses in some way avoid immune detection and cytolysis by anti-viral T cells (Joly et al., 1991; Griffin et al., 1992). The major mechanism by which neurons do so is the suppression of MHC class I molecules which are required for the recognition of viral peptides by cytolytic CD8⁺ T cells on virally infected cells (Oldstone et al., 1986; Joly et al., 1991; Joly and Oldstone, 1992). It has been previously demonstrated that the suppression of MHC class I molecules is neuron-specific because it is not seen in other CNS cell types including astrocytes or most cells of peripheral tissues (Drew et al., 1993; Massa et al., 1993). Further, the lack of expression in neurons is transcriptionally regulated. In particular, positive transcription factors, that bind to a κ B site and to the interferon regulatory factor element (IRF-E) (Watanabe et al., 1991; Kimura et al., 1994; Tanaka et

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al., 1994) present in the promoter region of this gene, are specifically absent in neurons (Drew et al., 1993; Massa et al., 1993). In general, κ B sites function by binding transcriptionally active dimers of the NF- κ B family of transcription factors (NF- κ B1 (p50), NF- κ B2, Rel, and RelA (p65)) (Lenardo and Baltimore, 1989; Ghosh et al., 1990; Baeuerle, 1991; Schreck et al., 1991; Beg and Baldwin, 1993; Nabel and Verma, 1993) and the IRF-E binds to closely related positive and negative transcription factors, including interferon regulatory factors (IRFs)-1 and -2 (Harada et al., 1989; Fu et al., 1992; Nelson et al., 1993; Pellegrini and Schindler, 1993; Tanaka et al., 1993). In addition to IRFs, the core IRF-E of some, but not all, interferon-inducible genes may have additional flanking sequences that are required for the binding of an additional factor termed interferon (IFN)- α -stimulated gene factor 3 (ISGF3), which is induced by IFN- α/β , but not IFN- γ . This extended sequence is called the interferon-stimulated responsive element (ISRE) (Darnell et al., 1994).

Both IFNs and viruses rapidly induce NF- κ B and IRF-E-binding activities in most cells and often account for both IFN- and virus-inducible expression of genes containing these enhancers (MacDonald et al., 1990; Xanthoudakis and Hiscott, 1990; Cohen and Hiscott, 1992; Thanos and Maniatis, 1992; Pellegrini and Schindler, 1993). Thus, both IRF-E and κ B sites are often virus-responsive elements (VREs) (Fan and Maniatis, 1989; MacDonald et al., 1990; Thanos and Maniatis, 1992; Du et al., 1993). This is apparent in the case of the MHC class I and the IFN- β gene which are coordinately up-regulated in most cells by juxtaposed κ B and IRF-E enhancers that interact primarily with either IFN- or virus-induced nuclear transcription factors NF- κ B (p50:p65) and IRF-1, respectively (Israel et al., 1986; Shirayoshi et al., 1988; Korber et al., 1988; Fujita et al., 1985; Fujita et al., 1988; Fujita et al., 1992; Burke and Ozato, 1989; Israel et al., 1989; Fan and Maniatis, 1989; Lenardo et al., 1989; Chang et al., 1992; Reis et al., 1992; Thanos and Maniatis, 1992; Du et al., 1993). Consistent with these observations is that induced IFN can further increase these transcription factors and lead to a secondary sustained autocrine induction, or priming for induction by virus, of IFN- β and MHC class I genes (Enoch et al., 1986; Fan and Maniatis, 1989; Dhib-Jalbut and Cowan, 1993).

The lack of MHC class I molecules on neurons protects these cells against immunopathological T cell responses during CNS viral infections (Joly et al., 1991). Therefore, mechanisms that control the cytopathic effects of viruses harbored within neurons are essential to neuronal survival (Schijns et al., 1991; Griffin et al., 1992). A rapid response in controlling viral infections is IFN produced by and acting on cells in the immediate vicinity of viral replication. However, because neurons

were previously shown to lack constitutive (Drew et al., 1993) or IFN- γ -inducible κ B- and IRF-E-binding factors (Massa et al., 1993) for the upregulation of MHC class I and IFN- β genes, it was hypothesized that neurons may be generally deficient in the expression of IFN- β and perhaps antiviral state genes since the latter commonly contain IRF-Es (Pellegrini and Schindler, 1993). Because of the potential importance of an IFN and anti-viral gene response within the CNS, an analysis of IFN- β , anti-viral state, and MHC class I induction in primary neuronal cultures following exposure of these cells to known IFN-inducing agents, including double-stranded RNA and virus, was undertaken. Unexpectedly, it was found that neurons rapidly express high levels of IFN- β , but not MHC class I molecules, following exposure to these inducing agents. Also, both production and response to IFN- β in neurons is unique in these cells. These studies were of particular interest mechanistically because unique selective transcriptional activation of IFN- β and anti-viral state genes, but not the MHC class I gene, is required in neurons.

2. Materials and methods

2.1. Neuron and astrocyte cultures

Neuronal cultures were produced from 8-day post-natal mouse cerebellum of B10.A mice as previously described (Meier and Schousboe, 1982; Massa et al., 1993). Neurons were transfected at 1 day after plating, treated with inducing agents 2 days after plating, and processed for either FACS analysis, Northern analysis of RNA, CAT assay or gel mobility shift assay at different timepoints thereafter. Astrocytes were prepared from newborn B10.A mice (Harlan-Sprague Dawley, Indianapolis, IN) as previously described (Massa et al., 1992; Massa et al., 1993) and the cultures were fed at 5 days post-plating. At 6 days after plating the cultures were transfected with CAT constructs, treated with inducers at 7 days, and were processed to either extract CAT enzyme, mRNA, or nuclear proteins at 8 days after plating.

2.2. MHC class I and IFN-inducing agents

The following agents were used: recombinant murine IFN- γ (a generous gift from Genentech, Inc., S. San Francisco, CA); natural murine IFN- α/β (Lee Biomolecular Research Laboratories, Inc., San Diego, CA); double-stranded poly(I):poly(C) (Pharmacia, Piscataway, NJ); and Sendai virus (SPAFAS, Storrs, CT). These were generally used at 100 U/ml for cytokines, 100 μ g/ml for poly I:poly C, and 500 hemagglutinating U/ml for Sendai virus unless indicated otherwise in the text.

2.3. Northern blot analysis

Total RNA was extracted from neuron and astrocyte cultures using a guanidine isothiocyanate technique (Chomczynski and Sacchi, 1987). 15 μ g of RNA from each specimen was electrophoresed in a 0.9% agarose gel and then transferred to a nylon filter (Nytran Plus, Schleicher and Schuell, Keene, NH). The RNA was hybridized with 32 P-labelled cDNA probes encoding either the conserved third external transmembrane and cytoplasmic region of the murine H-2L^d MHC class I molecule (pH-2^d-3) (Lalanne et al., 1982) or murine IFN- β (Higashi et al., 1983). Autoradiographs were quantified by densitometry.

2.4. Gel mobility shift analysis and oligonucleotide probes

Nuclear extracts from neurons and astrocytes were prepared using a mini-prep technique, as described previously (Lee et al., 1988; Massa et al., 1992). Protein determinations were performed using the Biorad protein assay kit (Biorad Laboratories, Richmond, CA). All oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer (Model 391-04), annealed to complementary sequences to produce double-stranded DNA probes, labeled, and gel purified. Duplex oligonucleotides with the following coding strand sequences were used:

- IFN- β PRDII κ B site: 5' GTGGGAAATCC 3' (Fan and Maniatis, 1989)
- IFN- β PRDI IRF-E site: 5' GGAGAAGT-GAAAGTG 3' (Fan and Maniatis, 1989)
- MHC-CRE region I κ B site: 5' GCTGGGGATTCC-CAT 3' (Burke et al., 1989)
- MHC-IRF-E site(ICS): 5' ATCTCCTCAGTTTCAC-TTCTGCA 3' (Flanagan et al., 1991)
- Ig κ chain κ B site: 5' CTCAACAGAGGGGACTT-TCCGAGAGGCCAT 3' (Picard and Schaffner, 1984)

Binding of nuclear proteins to duplex oligonucleotide probes was analyzed using the gel mobility shift assay (Fried and Crothers, 1981; Garner and Rezvin, 1981; Massa et al., 1993). DNA probes were prepared by end-labelling with [γ - 32 P]ATP using T4 polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, IN). The probes (20 000 cpm/ng DNA/reaction) were incubated with 10–20 μ g of nuclear extract in the presence of 0.5–1.0 μ g poly(dI:dC) (Pharmacia, Inc., Piscataway, NJ) for 20 min. To some reactions, various unlabelled competitor duplex oligonucleotides or antibodies to transcription factors were added before the labeled probe and further incubated for 40 min. The reaction mixtures were electrophoresed through a 4% polyacrylamide gel and autoradiographs of the gels were photographed.

2.5. Antibodies to transcription factors

Antibodies to IRF-1, IRF-2, and ISGF3 γ (Nelson et al., 1993) were whole rabbit polyclonal antisera (a gift from Dr. K. Ozato, NIH, Bethesda, MD) and were used at a concentration of 0.5 μ l/reaction. Affinity-purified rabbit IgG to p50 and p65 of NF- κ B were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used at a concentration of 2 μ g IgG/reaction.

2.6. Immunofluorescence staining for FACS analysis

Cultures were treated with cytokines and other agents for various amounts of time, briefly exposed to either trypsin (astrocytes) or dispase (neurons) to release single-cell suspensions from the dish, and stained with a rat monoclonal antibody to mouse MHC class I molecules (M1/42.3.9.8) (Springer, 1980). After incubation with goat anti-rat IgG conjugated to fluorescein isothiocyanate (FITC), the cells were analyzed by FACS (Becton Dickinson Immunocytometry Systems, Mountain View, CA) to determine the mean fluorescence intensities (MFI) of samples of 10 000 cells.

2.7. CAT assays and constructs

pIFN- β - and pL^d-CAT constructs listed in Fig. 1 were previously described and characterized (Miyazaki et al., 1986; Du and Maniatis, 1992; Nelson et al., 1993; Segars et al., 1993). The upstream promoter sequences in these constructs are connected to the chloramphenicol-acetyl-transferase (CAT) gene as a reporter of transcriptional activity (Gorman et al., 1982). Neurons and astrocytes were transfected using the DEAE-dextran method (McCutchan and Pagano, 1968). All cultures were also co-transfected with pCH-110, an SV40- β -galactosidase gene construct (Pharmacia, Inc., Piscataway, NJ) to standardize transfection efficiency between samples.

2.8. IFN and virus plaque reduction assay

NIH 3T3 cells or neurons were treated overnight with supernatants from poly I:poly C- or Sendai virus-treated neurons or control medium (neurons not treated with inducers). To some of the cultures, a neutralizing rat monoclonal antibody specific for mouse IFN- β (20 000 neutralizing U/ml) (Seikagaku America, Inc., Rockville, MD) was added with the supernatants. The next day, the cells were rinsed and inoculated with vesicular stomatitis virus (VSV) at a multiplicity of infection of 1. 18 h later, the virus was harvested, PFU/ml were quantified, and laboratory U/ml of IFN were determined (Grossberg et al., 1986) using NIH-3T3 cells.

3. Results

3.1. MHC class I and IFN- β expression in neurons and astrocytes

In the present study, it was of interest to determine whether the MHC class I and IFN- β genes were coordinately or independently regulated in neural cells using IFN inducers (Grossberg et al., 1986). As in other cell types, co-regulated expression of these genes was initially expected based on previous studies by others showing conserved juxtaposed enhancer sequences, κ B and IRF-E sites, common to the promoter of both genes (Fig. 1). Quantitative analysis of MHC class I molecules by immunofluorescence showed that astrocytes treated with either Sendai virus, poly I:poly C, or IFN- α/β were induced to express high levels of MHC class I molecules (Table 1). However, identical treatment of neurons with these agents did not induce detectable levels of MHC class I molecules (Table 1). Therefore, neurons appeared to be uniquely non-responsive to MHC class I-inducing agents as previously described for IFN- γ (Massa et al., 1993) and extended here.

In light of the above studies, it was of interest to determine whether IFN- or virus-inducible expression of the IFN- β gene was also absent in neurons. Northern blot analysis of MHC class I and IFN- β genes showed that both were coordinately inducible in astrocytes treated with the potent IFN inducer, poly I:poly C, as expected (Table 1). In contrast to astrocytes, only the IFN- β gene, but not the MHC class I gene, was induced to high levels in neurons (Table 1). Surprisingly, neurons expressed at least 100-fold more IFN- β messenger RNA than astrocytes similarly treated. This indicated that the IFN- β and MHC class I genes are differentially regulated in neurons. To further verify this observation and its functional importance, an analysis of IFN- β activity in neuronal culture supernatants following treatment with inducing agents was undertaken.

Table 1
FACS and Northern blot analysis of MHC class I and IFN- β induction

	Astrocytes				Neurons			
	C	IFN α/β	PI:PC	SV	C	IFN α/β	PI:PC	SV
FACS								
MHC class I ^a	8.1	14.5	14.5	26.1	1.0	1.4	1.2	1.0
mRNA								
MHC class I ^b	1.0	–	8.4	–	1.0	–	0.7	–
IFN- β ^b	1.0	–	5.8	–	1.0	–	548.0	–

^a Mean fluorescence intensity of 10000 cells above background value of 1.0.

^b Density of bands relative to control (C).

–, not determined; SV, Sendai virus; PI:PC, poly I:poly C.

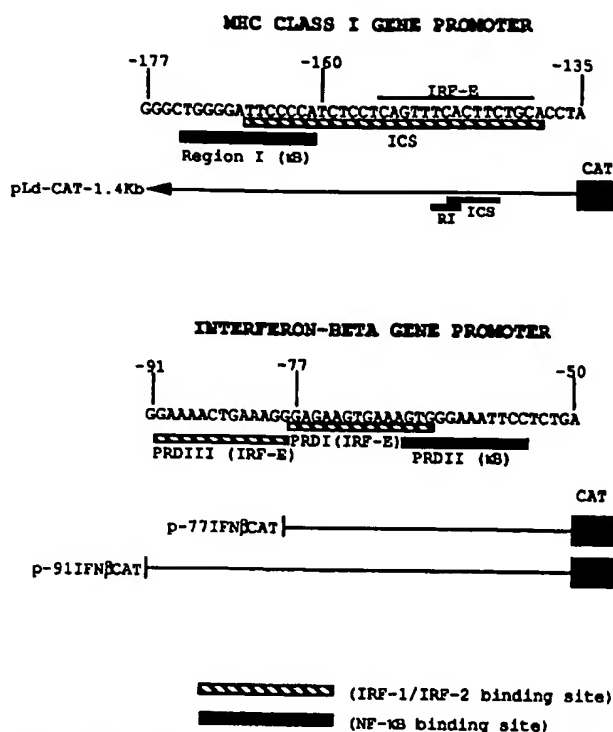


Fig. 1. MHC class I and IFN- β gene promoter regions indicating relevant cis-regulatory elements (κ B and IRF-E). Nucleotides are numbered with respect to transcription start sites. CAT constructs used for transfection experiments are indicated below each promoter element. IRF-1/IRF-2 and NF- κ B binding sites are indicated by striped and solid bars, respectively.

3.2. Identification of IFN- β anti-viral activity in neurons

A determination of IFN titers in culture supernatants from neurons treated with inducers was performed by VSV plaque reduction assay. In this assay, neurons were pulse-treated either with poly I:poly C or Sendai virus, rinsed, and 24 h later the resulting culture supernatants were analyzed for the presence of anti-viral (interferon) activity. This was done by incubating NIH 3T3 cells with the neuronal supernatants for 24 h to induce an antiviral state, after which the cells were infected with VSV. The ability of the supernatant-treated cells to produce viral particles was then quantified in PFU/ml and units/ml interferon were accordingly computed. As shown in Table 1, neurons produced anti-viral activity, which could be directly attributed to IFN- β using a neutralizing monoclonal antibody to murine IFN- β (Table 2, top). Therefore, neurons selectively activate the IFN- β gene and elaborate functional IFN- β .

3.3. Anti-viral, but not MHC class I-inducing, activity of neuron-derived IFN- β on neurons

It was of interest to determine whether neuronally derived IFN- β induced an anti-viral state in neurons.

Neurons treated with neuronal IFN- β produced several logs less virus compared to neurons treated with control neuronal supernatants (see PFU/ml (neurons), Table 2). However, as was found with murine IFN- α/β (Table 1), neuronal IFN- β did not induce MHC class I molecules on neurons (see MHC class I (neurons), Table 2). The latter was not related to an inherent difference in neuronal IFN- β from that produced from other sources, because neuronal IFN- β was able to induce MHC class I molecules on astrocytes (Table 2, bottom). This indicated that neurons selectively activated IFN- β gene expression when exposed to viral materials and responded selectively to the different functional activities of IFN- β .

3.4. Inducible promoter activity of the IFN- β gene in neurons

In order to determine whether the promoter of the IFN- β gene was inducible following treatment of neurons with poly I:poly C or Sendai virus, neurons were transfected with two IFN- β promoter constructs containing κ B and IRF-E elements upstream of the CAT reporter gene (Fig. 1). After transfection with p-77IFN- β CAT and p-91IFN- β CAT, the cells were treated with either poly I:poly C, Sendai virus, or IFN- γ . Fig. 2 indicates that the construct p-77IFN- β CAT containing PRDI (a canonical IRF-E) and PRDII (a κ B site) enhancers is sufficient for the induction of CAT activity following treatment of neurons with poly I:poly C or Sendai virus. Addition of PRDIII (a second juxtaposed IRF-E) in the p-91IFN- β CAT construct, did not significantly differ in CAT activity to that of p-77IFN- β CAT. IFN- γ did not induce these constructs (Fig. 2), which is consistent with the inability of IFN- γ to induce binding activities to these enhancer sites in neu-

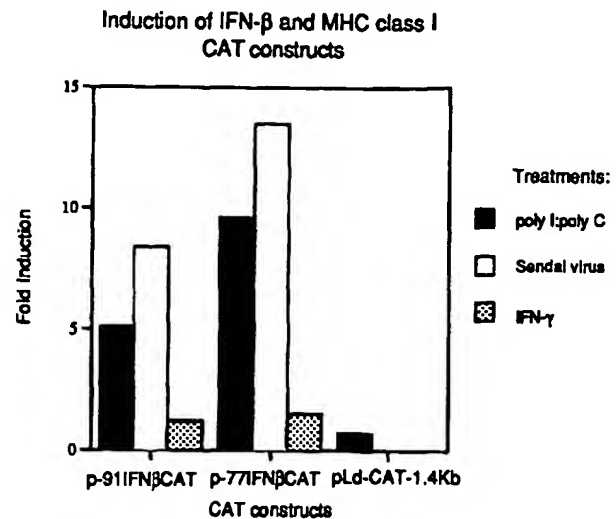


Fig. 2. CAT assays of neurons transfected with either p-77IFN β CAT, p-91IFN β CAT, or pLd-CAT-1.4 Kb. Primary cultures of neurons were transiently transfected with the constructs and subsequently treated with either poly I:poly C, Sendai virus, IFN- γ , or medium alone. Fold induction following treatment with these agents is relative to CAT levels in transfected cells treated with medium alone. Transfection efficiency was normalized with respect to β -galactosidase activity/unit protein. Experiments were repeated three times. Induced CAT activities of p-77IFN β -CAT and p-91IFN β were not significantly different (Student's *t*-test, *P* = 0.3).

rons as previously described (Massa et al., 1993). The induction of the pIFN- β CAT constructs was specific because treatment of neurons with poly I:poly C or Sendai virus did not induce a MHC class I CAT construct (Fig. 2) containing potentially functional IRF-E and κ B sites of this gene (Fig. 1). This indicated preferential transcriptional activation of the κ B and/or IRF-E sites in the IFN- β gene but not in the MHC class I gene.

Table 2
Analysis of IFN- β activity in neurons

Treatment	Neuronal supernatants ^a						
	PI:PC	SV	+ α IFN- β				
PFU/ml ^b	—	—	—	—	—	—	—
Units/ml IFN- β ^c	—	—	—	—	—	—	—
PFU/ml (neurons) ^d	—	—	—	—	—	—	—
MHC class I (neurons) ^e	—	—	—	—	—	—	—
MHC class I (astrocytes) ^e	—	—	—	—	—	—	—

^a Neuronal supernatants were produced by treating neuronal cultures with either PI:PC or Sendai virus (SV). Neutralizing antibody to IFN- β (+ α IFN- β) was added to some of the supernatants to assess the presence of induced IFN- β .

^b Titer of VSV produced from NIH 3T3 cells treated before infection with the different neuronal supernatants listed above.

^c Laboratory U/ml of interferon in the neuronal supernatants is based on PFU/ml reduction as the result of induced interferons compared to control supernatants (minus inducers, 1st column).

^d PFU/ml of VSV produced by neurons treated before infection with the above neuronal supernatants.

^e Values for MHC class I are induction indices for fluorescence intensity of cells treated with IFN- β -containing supernatants divided by fluorescence of cells grown in control medium. For determining IFN- β activities on neurons and astrocytes, IFN- β -containing supernatants were diluted to 10 U/ml and then added to the cultures with or without anti-IFN- β antibody.

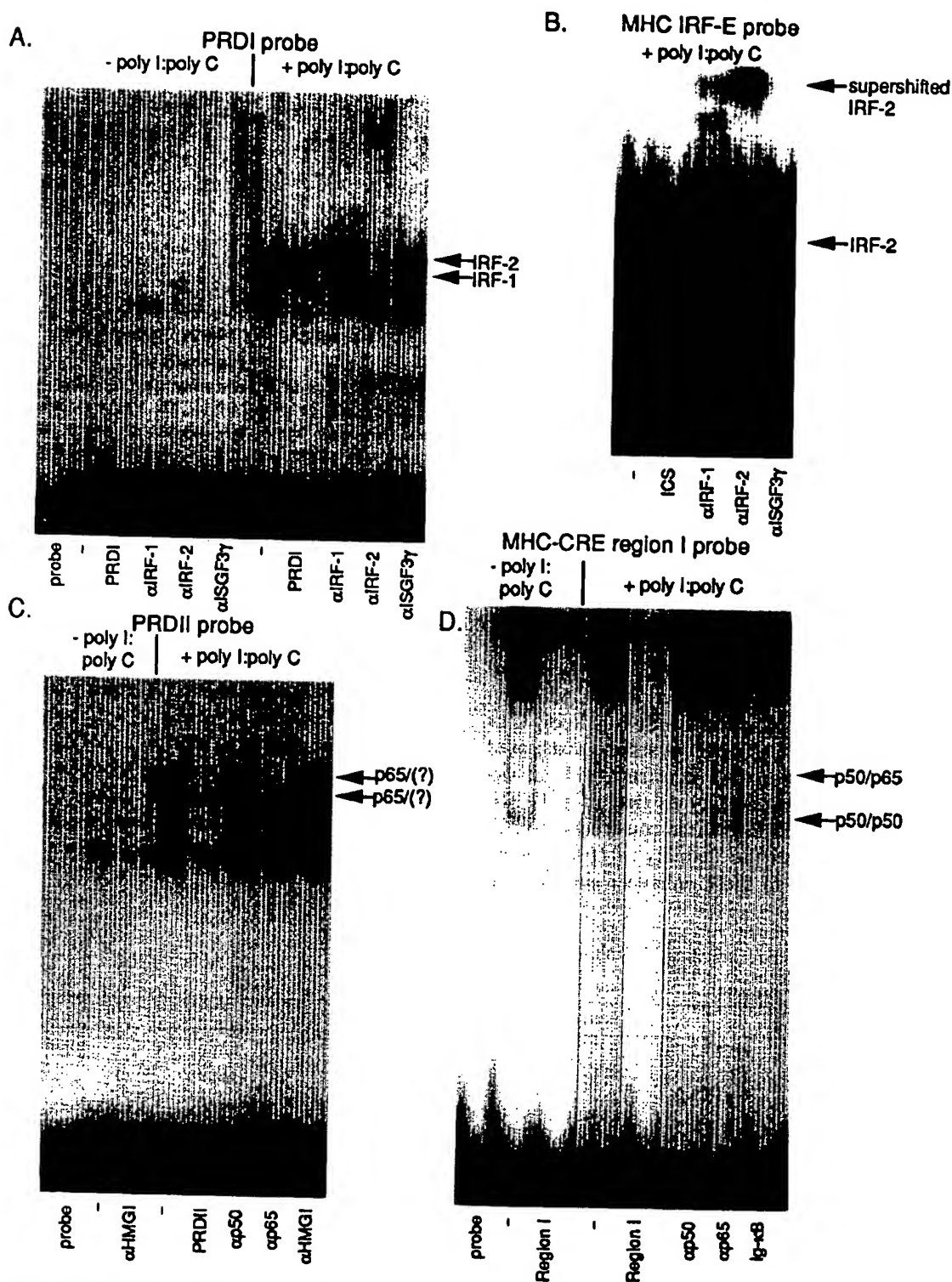


Fig. 3. Gel mobility shift assays of nuclear proteins extracted from neurons treated with either medium alone (-poly I:poly C) or medium containing poly I:poly C (+poly I:poly C). Extracts were probed with either PRDI (A), MHC class I IRF-E(ICS) (B), PRDII κ B site (C), or MHC-CRE region I κ B site (D) 32 P-labeled duplex oligonucleotides. Some bands in the gels were competed using a 100 \times molar excess of unlabeled oligonucleotides (PRDI, PRDII, ICS, Region I) or were supershifted using monospecific antibodies to transcription factors (α IRF-1, α IRF-2, α ISGF3 γ , α HMG1, α p50 NF- κ B, and α p65 NF- κ B) as indicated at the bottom of each gel. Each lane was loaded with a reaction mixture containing 15 μ g of nuclear protein and either 0.5 or 1 μ g of poly(dI:dC). Arrows indicate specific binding activities as labeled.

The IFN- β gene is regulated by two conserved juxtaposed enhancers designated PRDI (IRF-E site) and PRDII (κ B site). Similar juxtaposed sequences are present in the MHC class I promoter, designated the ICS (interferon consensus sequence) (IRF-E site) and the MHC-CRE region I (κ B site) (Figs. 1 and 4) (Burke and Ozato, 1989; David-Watine et al., 1990; Singer and Maguire, 1990; Ting and Baldwin, 1993). A number of studies indicate that transcriptional enhancement through either interactions of IRF-1 with the IRF-E (Pine, 1992) or of different NF- κ B species at the κ B sites are sufficient for up-regulation of MHC class I and IFN- β genes and that synergy between the two sites may occur (Shirayoshi et al., 1988; Du et al., 1993). Previous work in this laboratory indicated that lack of MHC class I gene expression in neurons is related to the lack of these transcription factors binding to the MHC class I gene promoter (Massa et al.,

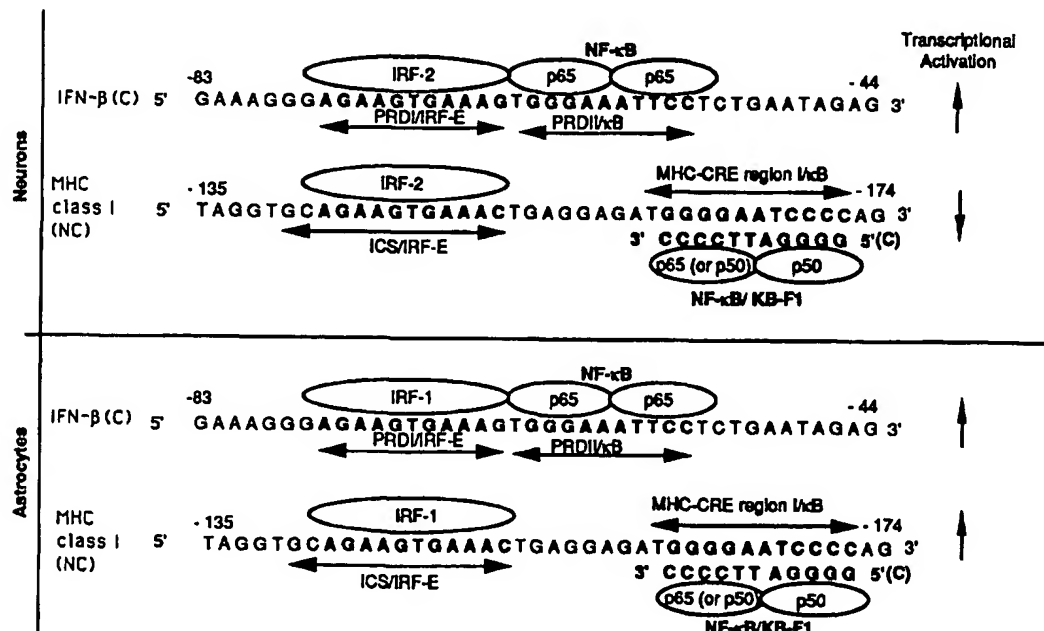


Fig. 4. Hypothesis of unique non-coordinate expression of the IFN- β and MHC class I genes in neurons and comparison with coordinate expression in astrocytes as outlined in the text. NF- κ B p65 homodimers in neurons bind to and activate the IFN- β but not the MHC class I gene. Note that the IRF-E of both genes primarily binds IRF-2 in neurons as presented here, but to IRF-1 in astrocytes (Massa, P.T. and Wu, H., submitted) the latter which accounts for MHC class I expression.

1993) and led to the question of whether the IFN- β gene was similarly silenced in these cells. Surprisingly, it was found that the IFN- β gene was highly expressed in neurons after treatment with poly I:poly C or Sendai virus while the MHC class I gene remained silent. Further, neurons responded differentially to treatment with neuronally derived IFN- β by expressing an anti-viral state, but not MHC class I genes. These properties may represent a mechanism whereby neurons successfully escape MHC class I-restricted lysis by cytolytic T lymphocytes during viral infection (Joly et al., 1991), while concurrently halting production and spreading of viral particles to other neurons (Schijns et al., 1991). This study was aimed at determining the selectivity of expression of these genes in neurons.

The apparent paradox of activation of the IFN- β , but not the MHC class I gene, in neurons prompted an analysis of κ B and IRF-E enhancer function and enhancer-binding activities in neurons. CAT assay data utilizing IFN- β and MHC class I promoter region constructs indicated that the κ B and/or IRF-E binding site of the IFN- β , but not the MHC class I, gene are responsible for inducible activity. The importance of the IFN- β κ B site and its strength relative to the MHC class I κ B site in neurons is consistent with recent CAT analyses showing that point mutation of the IFN- β site to that of the MHC class I sequence sharply reduces both NF- κ B binding and virus (or dsRNA) induction of IFN- β constructs (Lenardo et al., 1989; Thanos and Maniatis, 1992). With the functional CAT data, gel shift analysis also suggested that the κ B site (PRDII), rather than the two IRF-E sites (PRDI and PRDIII), may play the major role in the induction of the IFN- β gene. This is consistent with present data showing that when neuronal extracts were probed with PRDI, the predominant activity detected was the IRF-E transcriptional antagonist IRF-2. Conversely, when neuronal extracts were probed with the PRDII duplex oligonucleotide, three inducible bands were evident, two of which were supershifted with anti-p65, but not anti-p50 antibodies. Presently it is believed that the prevalence of p65 (RelA) binding activity to PRDII in neuronal extracts is consistent with high transcriptional activity, especially of p65₂ homodimers, at PRDII (Fujita et al., 1992; Kunsch et al., 1992; Perkins et al., 1992; Duckett et al., 1993; Ganchi et al., 1993).

IRF-1 is a transcriptional activating factor and the related factor, IRF-2, antagonizes the binding and transcriptional activity of IRF-1. Because inducible IRF-2 predominated over the expression of IRF-1 in neurons, it is proposed that IRF-E sites, PRDI and PRDIII of the IFN- β gene and the ICS of the MHC class I gene, are functionally silenced in neurons. This was supported by CAT assay of these sites in neurons. For instance, addition of PRDIII in the p-91IFN- β CAT construct, which is a functional IRF-E in other

cell types (Goodbourn et al., 1985), showed no significant difference in CAT activity compared to the p-77IFN- β CAT construct. Further, the MHC class I construct which contains a functional IRF-E (Shirayoshi et al., 1988) was not inducible in neurons, but is inducible in astrocytes (Massa et al., 1992; Massa et al., 1993). Collectively, these data indicate that PRDII may be the only functional enhancer of the IFN- β gene in neurons. The ability of the PRDII κ B site to act as a sole virus-inducible enhancer is indicated by reports showing that deletion constructs destroying the 5' end of PRDI, but not PRDII (to -73 relative to the start site), mediated a 15-fold induction of the gene in mouse fibroblasts (Zinn et al., 1983; Goodbourn et al., 1985). More directly, Visvanathan and Goodbourn (1989) showed that poly I:poly C induced large amounts of NF- κ B which independently mediated IFN- β gene induction through a single PRDII κ B site, without juxtaposed IRF-Es. Finally, an important role for PRDII is consistent with recent reports showing that targeted disruption of the IRF-1 gene in mice does not appreciably affect the induction of type I interferon genes by virus or double-stranded RNA (Matsuyama et al., 1993; Reis et al., 1994), agents that are potent inducers of both IRF-1 and NF- κ B factors (Grilli et al., 1993).

It has been shown that induction of anti-viral state by interferons or directly by viral materials (Wathelet, 1992) may involve the expression of positively acting transcription factors IRF-1 and/or interferon-stimulated gene factor-3 (ISGF3) acting through the approximately 15 base pair ISRE in anti-viral state genes (Pine, 1992; Reis et al., 1992; Darnell et al., 1994; Kimura et al., 1994). However, because the central 9-bp core of ISREs are IRF-Es (Pellegrini and Schindler, 1993), inducible IRF-2 in neurons may occupy and repress ISREs, indicating that the mechanism of induction of antiviral state genes may be ISRE-independent in these cells. Even though the MHC class I IRF-E is reportedly a potential ISRE (Pellegrini and Schindler, 1993) and the present study has been unable to detect ISGF3 at any timepoint with this probe, an analysis of a number of other known canonical ISREs of antiviral state genes must be performed to determine whether ISGF3 is rapidly inducible from pre-formed components (Darnell et al., 1994), even in trace amounts in neurons. On the tentative conclusion that neurons may lack ISGF3, anti-viral state genes in neurons may be under the control of interferon or virus response elements distinct from those that regulate MHC class I genes, including γ -activation sites (GAS) (Decker et al., 1991; Sims et al., 1993) and/or κ B sites. With respect to the former, IFN- α/β appears to induce GAS binding activities both similar and unique to those induced by IFN- γ in neurons (unpublished observations).

The present report describes both the specific activation of the IFN- β gene and the selective response to IFN- β activities in neurons. This selectivity is consistent with present concepts on mechanisms of neuronal survival related to CNS immunoprivilege. Thus, agents, including viruses, that commonly induce a set of genes containing κ B and IRF-E sites or VREs (Fan and Maniatis, 1989; MacDonald et al., 1990; Thanos and Maniatis, 1992; Du et al., 1993), specifically activated the IFN- β gene, but not the MHC class I gene. Further, neuronally derived IFN- β induces an antiviral state in neurons but not MHC class I molecules in distinction to other cells, including astrocytes. It is currently believed that site-specific interactions of virus- or IFN- β -inducible transcription factors, especially p65-containing NF- κ B species and IRF-2, may be the major mechanism for differential control of these genes in neurons, as summarized in Fig. 4. Overall, the present data demonstrate a specialization whereby certain cells may survive viral infections by both avoiding cytopathic effects of viruses (anti-viral state) and evading immunopathogenic anti-viral cellular immune responses by silencing MHC class I genes. Within the CNS, this may be a specific adaptation of neurons because other cells in the CNS, including astrocytes, are not endowed with this specialization. It is proposed that the relative functional importance and regenerative capacity of neurons and astrocytes reflect the expression of this specialization.

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HOW CELLS RESPOND TO INTERFERONS

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ABSTRACT

Interferons play key roles in mediating antiviral and antigrowth responses and in modulating immune response. The main signaling pathways are rapid and direct. They involve tyrosine phosphorylation and activation of signal transducers and activators of transcription factors by Janus tyrosine kinases at the cell membrane, followed by release of signal transducers and activators of transcription and their migration to the nucleus, where they induce the expression of the many gene products that determine the responses. Ancillary pathways are also activated by the interferons, but their effects on cell physiology are less clear. The Janus kinases and signal transducers and activators of transcription, and many of the interferon-induced proteins, play important alternative roles in cells, raising interesting questions as to how the responses to the interferons intersect with more general aspects of cellular physiology and how the specificity of cytokine responses is maintained.

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INTRODUCTION

Type I (predominantly α and β) and type II (γ) interferons (IFNs) signal through distinct but related pathways. Enormous progress has been made in recent years in understanding how cells respond to IFNs, especially in uncovering the pathways that mediate inducible gene expression. We now know that these pathways involve (a) specific type I and type II receptors, which bind to the Janus kinases (JAKs), and (b) the signal transducers and activators of transcription (STATs), which in turn propagate the signals. Moreover, JAKs and STATs, discovered through investigations of IFN signaling, also are involved in many different cytokine- and growth factor-mediated pathways. We know of four mammalian JAKs and seven STATs. Several recent reviews describe signaling by IFNs in relation to other cytokines and growth factors (1–5) and more general aspects of JAK-STAT function and the family relationships (6–10).

After activation by JAKs through phosphorylation of a specific tyrosine residue, STATs form homo- or heterodimers through mutual phosphotyrosine-Src homology region 2 (SH2) interactions. STAT dimers bind to gamma-activated sequence (GAS) elements, which drive the expression of nearby target genes. Different GAS elements prefer different STAT dimers, helping to establish specificity. Both STAT1-2 heterodimers and STAT1 homodimers bind to p48, a member of the interferon regulatory factor (IRF) family. The resulting trimers—called IFN-stimulated gene factor 3 (ISGF3) in the case of the STAT1-2 heterodimer—bind to IFN-stimulated regulatory elements (ISREs) that are distinct from the GAS elements. ISREs drive the expression of most IFN α/β -regulated genes and a few IFN γ -regulated genes. This review describes the signaling pathways used to turn the IFN responses on and off and the functions of the induced proteins in mediating the major cellular responses to IFN. Many of the proteins involved in both signaling and responses have important alternative functions, which are also reviewed.

SIGNALING PATHWAYS

Interferon γ

The proximal events of IFN γ signaling require the obligatory participation of five distinct proteins: type I integral membrane proteins IFNGR1 and IFNGR2 (the subunits of the IFN γ receptor) and JAK1, JAK2, and STAT1 (2, 11). Recent work has revealed that this signaling pathway is necessary, though not always sufficient, for induction of most if not all IFN γ -dependent biological responses in vitro and in vivo. IFN γ receptors are expressed on nearly all cell types, with the possible exception of mature erythrocytes, and display strict species specificity in their ability to bind IFN γ (12). Functionally active IFN γ receptors consist of at least two species-matched polypeptide chains (Figure 1). IFNGR1 (previously the α chain or CD119w), a 90-kDa polypeptide encoded by genes on human chromosome 6 and murine chromosome 10, plays important roles in mediating ligand binding, ligand trafficking through the cell, and signal transduction (11, 12). IFNGR2 (previously the β chain or accessory factor-1),

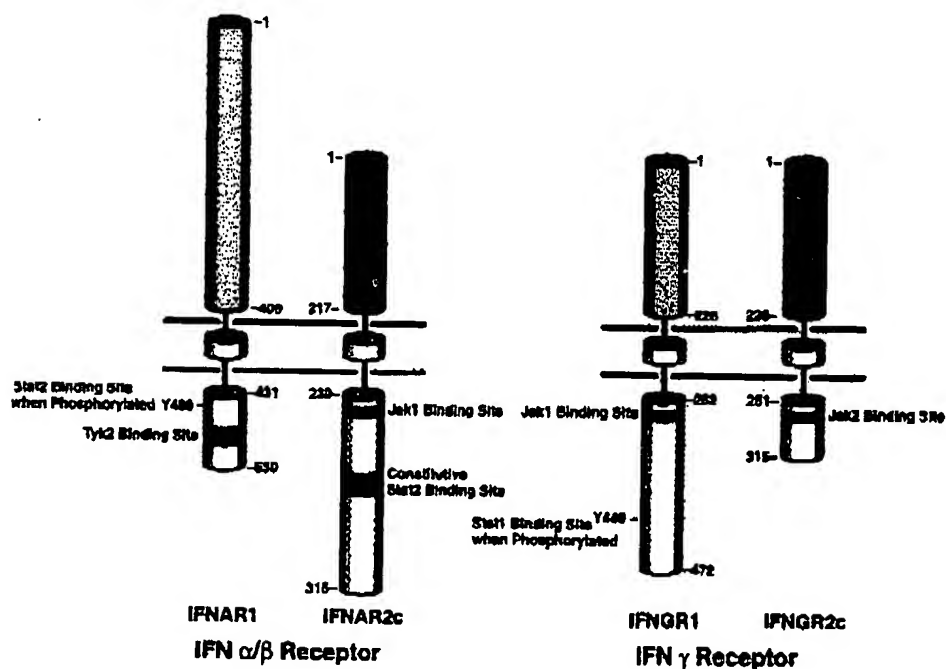


Figure 1 Schematic diagram of the human interferon (IFN) α/β and IFN γ receptors. (*left*) The IFNAR1 and IFNAR2c subunits of the IFN α/β receptor. (*right*) The IFNGR1 and IFNGR2 subunits of the IFN γ receptor. The positions of amino acid residues are shown inside each subunit, and functionally important intracellular domains are also identified. STAT: signal transducer and activator of transcription; JAK: Janus kinase.

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a 62-kDa polypeptide encoded by a gene on human chromosome 21 and murine chromosome 16, plays only a minor role in ligand binding but is required for signaling (11, 13, 14).

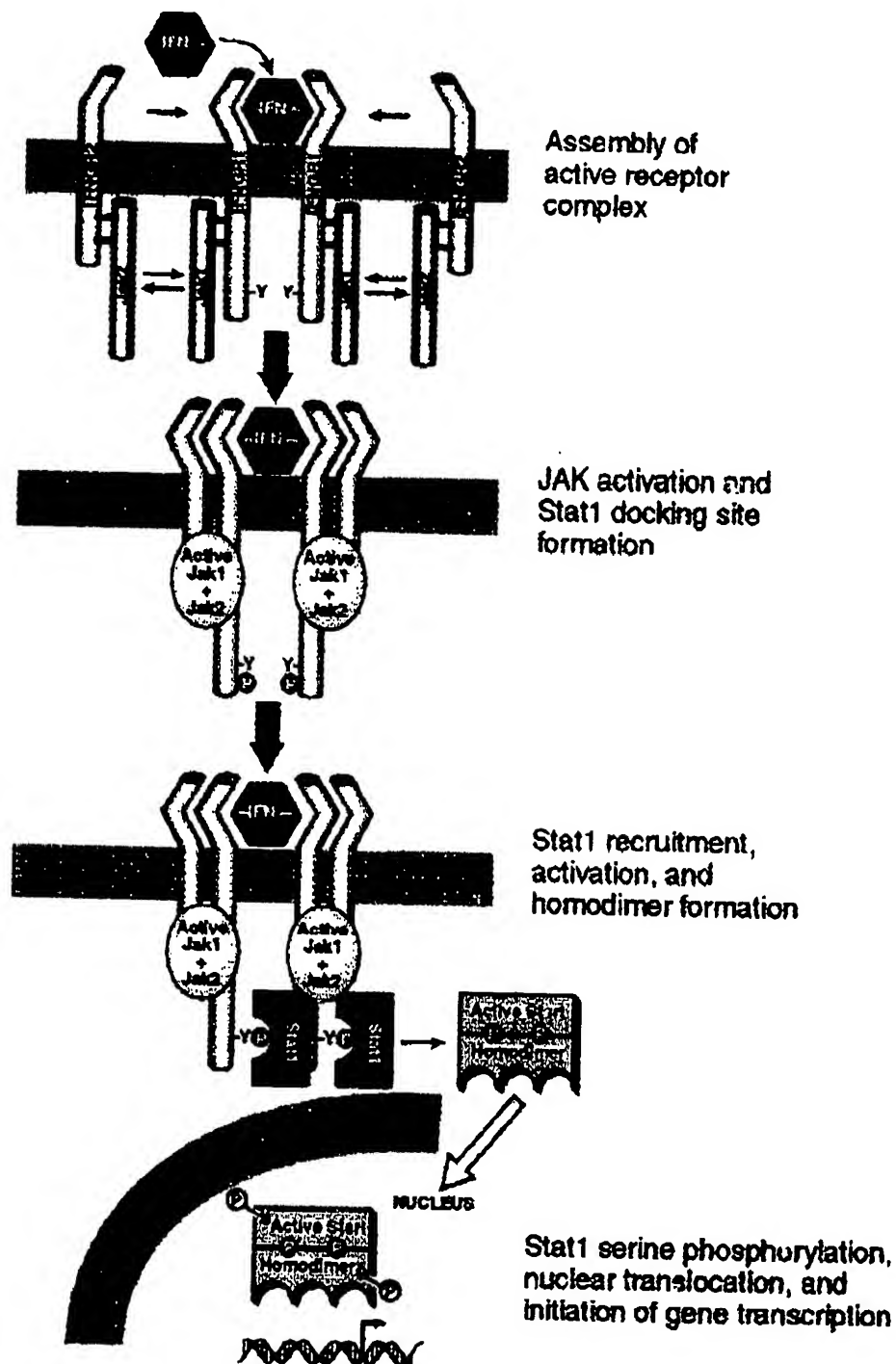
Three sets of experiments have implicated JAKs and STATs in mediating IFN γ -dependent cellular responses. First, isolation and complementation of mutant human cell lines have revealed that JAK1 and JAK2 become selectively activated in IFN γ -treated cells and are required for the ligand-dependent activation of IFN γ -inducible target genes (1). Second, through biochemical approaches, STAT1—a novel latent cytosolic transcription factor—was isolated and shown to undergo rapid tyrosine phosphorylation and activation in IFN γ -treated cells (1, 2). Third, structure-function analyses of the intracellular domains of the two IFN γ receptor subunits identified constitutive, specific binding sites for JAK1 and JAK2. Moreover, IFN γ induced the formation of a specific phosphotyrosine binding site on the receptor for STAT1, thereby providing the mechanism linking the activated receptor to its signal transduction apparatus (11).

Based on these and other observations, a relatively complete model of IFN γ signaling has been formulated (Figure 2). In unstimulated cells, the IFN γ receptor subunits do not preassociate with one another strongly (15), but their intracellular domains associate specifically with JAK1 and JAK2 (15–18). JAK1 binds to IFNGR1 through a 4-residue sequence (₂₆₆LPKS₂₆₉) in the membrane-proximal region of the IFNGR1 intracellular domain. JAK2 binds to a 12-residue, proline-rich Box 1-like sequence (₂₆₃PPSIPLQIEEYL₂₇₄) in the membrane-proximal region of the intracellular domain of IFNGR2.

Functionally active IFN γ is a homodimer that binds to two IFNGR1 subunits, thereby generating binding sites for two IFNGR2 subunits (15, 19–22). Within the resulting symmetrical signaling complex, the intracellular domains of the receptor subunits are brought into close proximity, together with the inactive JAKs that they carry. JAK1 and JAK2 are then sequentially activated by auto- and transphosphorylation. Activation of JAK2 occurs first and is needed for the subsequent activation of JAK1, which has a structural as well as enzymatic role (23). Work with chimeric JAK1 proteins and receptors has shown that the

Figure 2 Signaling through the interferon (IFN) γ receptor. The details of this model are described in the text. In unstimulated cells, IFNGR1 associates with Janus kinase (JAK)1, and IFNGR2 associates with JAK2. IFN γ induces oligomerization of the IFN γ receptor subunits, which leads to the transphosphorylation and activation of JAK1 and JAK2. The activated JAKs then phosphorylate Y440 of IFNGR1, creating a docking site for signal transducer and activator of transcription (STAT) 1. While bound to the receptor, STAT1 is phosphorylated on Y701 and is released from the receptor, forming a homodimer that translocates to the nucleus.

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specificity of JAKs lies in their capacity to associate with particular cytokine receptor subunits rather than because of a high degree of substrate specificity (24, 25).

Once activated, the receptor-associated JAKs phosphorylate a functionally critical, tyrosine-containing five-residue sequence ($_{440}\text{YDKPH}_{444}$) near the C terminus of IFNGR1, thereby forming paired ligand-induced docking sites for STAT1 (26–28). Two latent STAT1 proteins then bind to these sites because the SH2 domain of each recognizes the tyrosine-phosphorylated YDKPH sequence (28, 29). The receptor-associated STAT1 proteins are thus phosphorylated by the receptor-bound kinases at tyrosine 701, near the C terminus (30–32). The phosphorylated STAT1 proteins dissociate from the receptor and form a reciprocal homodimer, which translocates to the nucleus by a mechanism dependent on the GTPase activity of Ran/TC4 (33). The active STAT1 homodimers bind to specific GAS elements of IFN γ -inducible genes (1, 2) and stimulate their transcription. The transcriptional activity of STAT1 homodimers is enhanced at some point in the activation cascade by serine phosphorylation (at position 727) by an enzyme with MAP kinase-like specificity (34, 35).

Thus, the biological responses of cells to IFN γ result from ligand-dependent, affinity-driven assembly of a multimolecular signal transduction complex that derives at least some of its specificity from selective recruitment of only one member of the STAT family to its ligand-induced, tyrosine phosphate-docking site on the receptor. Importantly, subsequent work in other labs has shown that other members of the STAT family are recruited to their respective cytokine receptors by similar ligand-induced mechanisms. As a result, the IFN γ signaling model is now an accepted paradigm that explains an important mechanism of how cytokine receptors are coupled to their specific STAT signaling systems.

Processes that negatively regulate IFN γ signaling are only now being defined. In certain cells, such as T cells, IFN γ can induce desensitization by down-regulating the expression of the IFNGR2 mRNA and protein (36, 37). However, whether this mode of desensitization occurs in other cell types remains unclear. Dephosphorylation of the activated IFNGR1 subunit occurs rapidly following stimulation with IFN γ (26–27). However, because no data suggest that the IFN γ receptor associates with a particular phosphatase, dephosphorylation of the receptor may result from the action of general cellular phosphatases. More work in this area is needed. IFN γ (as well as several other cytokines) can induce the expression of a family of proteins termed SOCS/JAB/SSI, which bind to and inhibit activated JAKs (38–40). This work reveals that cytokines can desensitize cells in either a homologous or a heterologous manner by inducing proteins that block JAK activity. Although some of these proteins have been shown to inhibit IFN γ -induced biological responses when overexpressed

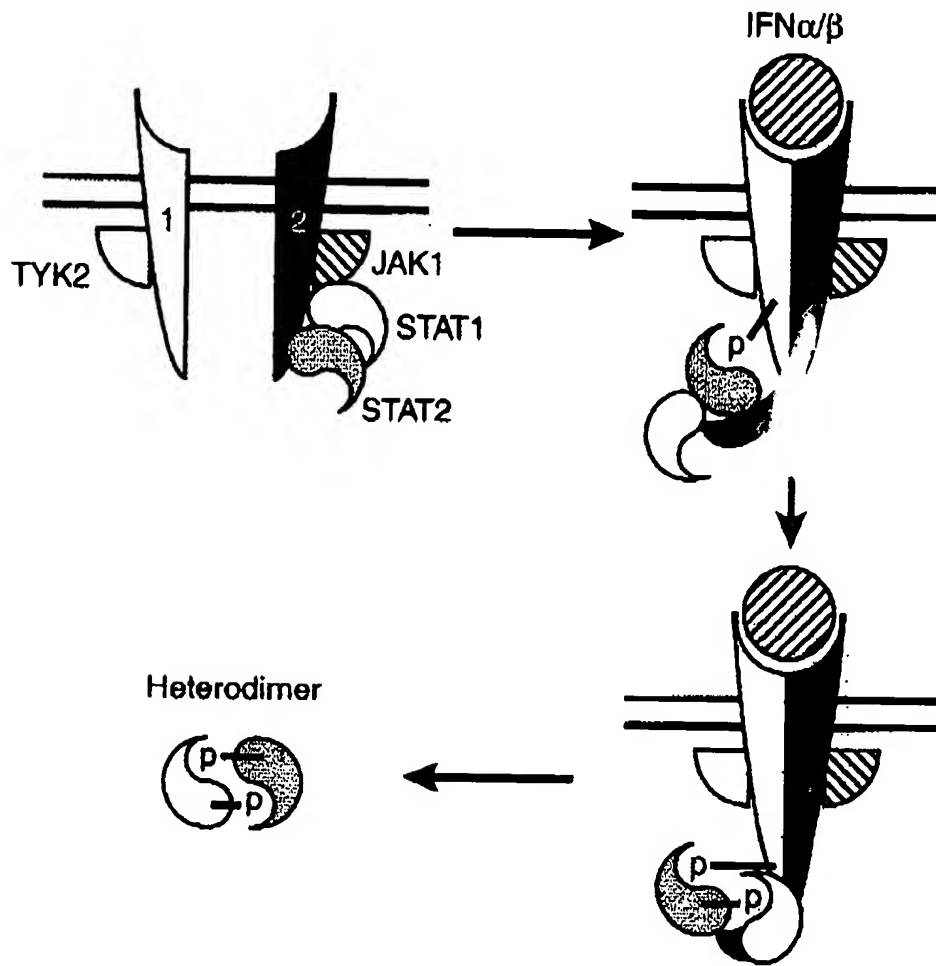


Figure 3 A model for the ordered formation of signal transducer and activator of transcription (STAT) 1 and STAT2 heterodimers at the interferon (IFN) α/β receptor. In the unliganded receptor, IFNAR1 associates with Tyk2, and IFNAR2 associates with Janus kinase (JAK) 1, STAT1, and STAT2. The binding of STAT1 to IFNAR2 depends on STAT2 but not vice versa. The IFN-mediated association of IFNAR1 and -2 facilitates the cross-phosphorylation and activation of Tyk2 and JAK1, which in turn phosphorylate Y466 of IFNAR1, creating a docking site for the SH2 domain of STAT2. This new interaction positions STAT2 for phosphorylation on Y690, thus creating a docking site for the SH2 domain of STAT1, positioning it for phosphorylation on Y701. Release of the STAT1-2 heterodimer from the receptor follows.

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complexed with one another, and then manipulation of those structures to reveal the detailed interactions that are crucial for function.

The overall plan of IFN α/β signaling (Figure 3) involves five major steps: (a) IFN-driven dimerization of the receptor outside the cell leads to (b) initiation of a tyrosine phosphorylation cascade inside the cell, resulting in (c) dimerization of the phosphorylated STATs, activating them for (d) transport into the nucleus, where they (e) bind to specific DNA sequences and stimulate transcription. Current understanding of this initial part of the response is greater than of the full response, which additionally involves the suppression of IFN-stimulated genes (ISGs) in the absence of IFN and down-regulation of the initial response in the continued presence of IFN. In addition to this main pathway, IFN α/β activates several other pathways. Although the biochemical evidence for additional signaling is persuasive, unfortunately we still have little knowledge of the physiological roles. It is also clear that different IFN α/β subtypes can stimulate distinct and different ancillary responses. As discussed below, the mechanism probably involves a novel pathway in addition to the one shown in Figure 3.

The receptor has two major subunits (Figure 1): IFNAR1 (the α subunit in the older literature) and IFNAR2c (the β_L subunit). IFNAR2a is a soluble form of the extracellular domain of the IFNAR2 subunit (48), and IFNAR2b (also called the β_S subunit) is an alternatively spliced variant with a short cytoplasmic domain (48) that, when overexpressed, can have dominant negative activity (49). Only IFNAR2c restores IFN α/β signaling to a mutant cell line in which the IFNAR2 gene has been inactivated (50). In contrast to the situation for the IFN γ receptor, neither IFNAR1 nor IFNAR2 alone binds to IFN α/β with the high affinity of the two-subunit combination (51,52). After IFN α/β is bound, the cascade begins with the phosphorylation of Tyk2, which is preassociated with IFNAR1 (53,54). JAK1, bound to IFNAR2c, can phosphorylate and activate Tyk2 (55), which can then cross-phosphorylate JAK1 to activate it further. Tyk2 also plays a structural role because the amount of IFNAR1 is low in Tyk2-null cells (56). The domains required for this role are distinct from those required to transduce the signal (57). Activated JAK1 and Tyk2 are almost certainly responsible for the sequential phosphorylation of Y466 of IFNAR1 (58), Y690 of STAT2, and Y701 of STAT1.

As shown in Figure 3, both STAT1 and STAT2 preassociate with IFNAR2c in untreated cells (59). STAT2 binds in the absence of STAT1, but STAT1 binds well only to the IFNAR2c-STAT2 complex (59). STAT1 and STAT2 also seem to associate with each other in the cytosol of untreated cells (60), but the physiological significance of this interaction is unclear. When Y466 of IFNAR1 is phosphorylated, the SH2 domain of STAT2 binds to it (61), followed by the phosphorylation of both STATs and dissociation of the phosphorylated heterodimer from the receptor.

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in cells, little information is available to define their enzyme or cytokine specificities. Nevertheless, further investigation of those novel proteins is likely to produce new insights into how JAK-STAT pathways are regulated.

The physiological relevance of IFN γ signaling through the JAK-STAT pathway and the basis for signaling specificity has been established unequivocally through the generation and characterization of mice with a targeted disruption of the STAT1 gene (41,42). STAT1-null mice show normal tissue and organ development, produce normal numbers and distributions of immune cell populations, and are able to reproduce. However, cells from these mice are incapable of manifesting any biologic responses to either IFN γ or IFN α , and the mice display severe defects in the ability to resist microbial and viral infections. In contrast, STAT1-null mice do not display abnormalities in responses induced by a variety of other cytokines [such as growth hormone, epidermal growth factor (EGF), and interleukin (IL)-10] that have been shown to activate STAT3 and STAT1 *in vitro*. Taken together, these results show that under physiological conditions, the development of biological responses induced by IFN γ (and in many cases also by IFN α/β) requires the participation of STAT1. These results further suggest that the use of STAT1 in signaling pathways under physiological conditions is restricted largely to the IFN systems. Thus, the specificity of IFN γ signaling is due predominantly to two temporally and topographically distinct processes involving STAT1. The first process is the recruitment of STAT1 to a specific docking site formed on the activated receptor at the membrane. In the second process, activated STAT1 dimers, once they arrive in the nucleus, activate a distinct set of cytokine-inducible genes.

The ability of STAT1 to activate gene expression may also be modulated by its interaction with other transcription factors. For example, IFN γ -dependent induction of the 9-27 gene is mediated by the interaction of a STAT1 homodimer-p48 complex with an ISRE rather than with a GAS element (43,44). In addition, induction of the ICAM-1 gene by IFN γ depends on the interaction of STAT1 and the transcription factor Sp1, which occurs when both proteins are bound to DNA (45). Thus, cell type-specific gene induction by IFN γ may be explained, at least in part, by the ability of additional cell-specific positive and negative factors to modulate the actions of STAT1 (46,47).

Interferons α and β : The Common Pathways

The main pathway of response to IFN α/β requires two receptor subunits, two JAKs, two STATs, and the IRF-family transcription factor p48 (Figure 3). The IFN α/β signaling pathways are understood at least as well as any other, but we are still at a relatively early stage, capable of drawing blobs to illustrate the major interactions but ignorant of the fine mechanistic detail. It will require analysis of the three-dimensional structures of the major components, individually and

Experiments in which the SH2 domains of STAT1 and STAT2 have been interchanged reveal that the specificity of the IFN γ receptor for STAT1 requires the SH2 domain (29) because a variant STAT1 carrying the SH2 domain of STAT2 does not function. However, in the case of the IFN α/β receptor, a very different result is obtained. STAT2 works equally well with either its own SH domain or that of STAT1 (29, 59), revealing that different domains of STAT2 are more important in establishing specific interactions with the receptor. The N-terminal third of STAT2 has been identified as the dominant subregion in determining specificity (59).

In addition to the seven major components discussed above, there is evidence that the tyrosine phosphatase SHP-2 may also be required for signaling. This enzyme preassociates with IFNAR1 and is phosphorylated in response to IFN α/β (62). In transient cotransfection experiments, a dominant negative form of SHP-2 inhibits the IFN α/β -induced expression of a reporter gene (62). Experiments showing that SHP-2 is required for IFN α/β -dependent activation of endogenous genes in untransfected cells would help to complete this interesting story.

How activated STATs reach the nuclei of IFN-treated cells is not yet clear. ISGF3, the major transcription factor formed in response to IFN α/β (Figure 4), is required to drive the expression of most ISGs via their ISREs, as shown by the specific defects in p48-null human cells (63, 64). Interestingly, p48-null mice also show severe defects in the induction by viruses of the IFN α/β genes themselves, consistent with the binding of ISGF3 to virus-inducible elements within the IFN α/β promoters (65). STAT1-2 heterodimers and STAT1 homodimers form in response to IFN α/β independently of p48, and each can drive the expression of a minority of ISGs, such as the IRF1 gene, through GAS elements (66, 67). The relative amounts of STAT1-2 heterodimer and ISGF3, or of STAT1 homodimer and its complex with p48 (64), will obviously depend on the levels of p48, which can vary widely among different cell types. Because the STAT1 homodimer that forms in response to IFN α/β does not drive the expression of IFN γ -responsive genes that contain GAS elements, it stands to reason that an additional response to IFN γ is required and that a secondary modification of STAT1 homodimers in response to IFN γ may be involved (66). A prime candidate is the phosphorylation of serine 727 of STAT1 (34).

Initial analysis of the interaction of ISGF3 with the 6-16 and 9-27 ISREs showed that the protected region is about 35 nucleotides long (68, 69). The most meaningful contacts between the ISRE and ISGF3 involve STAT1, with p48 playing a less important role and STAT2 serving to provide a potent trans-activation domain (70, 71). The region between residues 400 and 500 of STAT1 provide binding-site specificity (72), and the region between residues 150 and 250 is involved in contacting the C-terminal portion of p48 (73). The STAT

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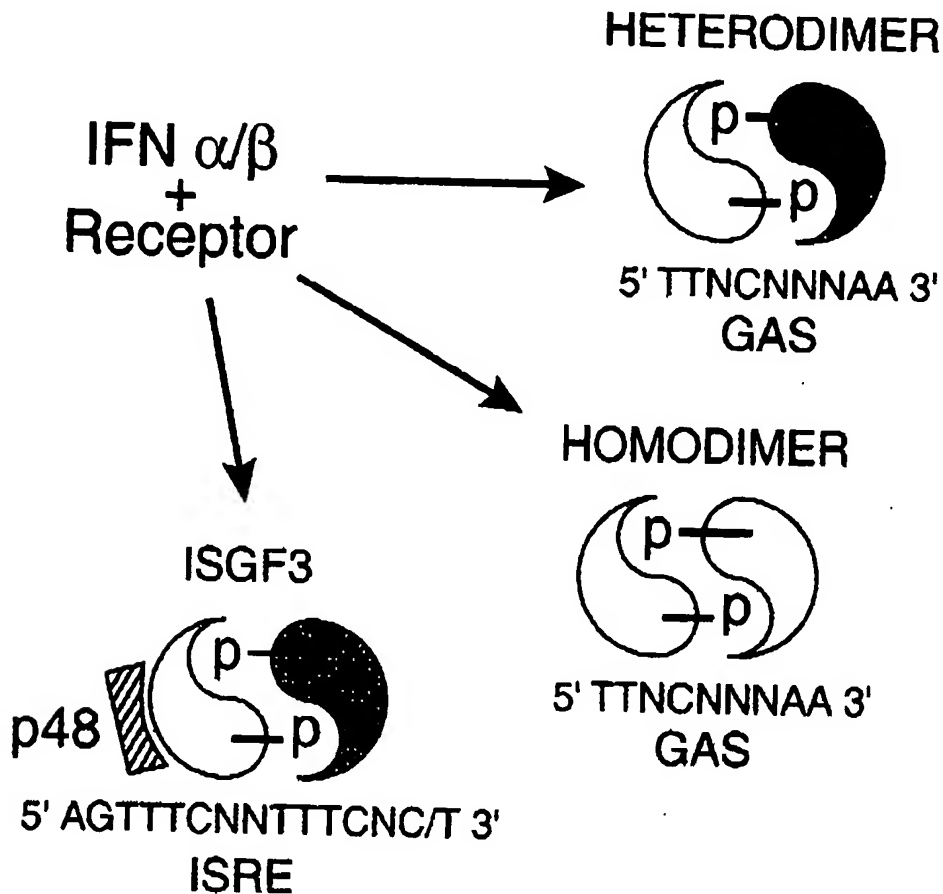


Figure 4 Transcription factors formed at the liganded interferon (IFN) α/β receptor and their DNA recognition elements. Signal transducer and activator of transcription (STAT) 1 and STAT2 heterodimers and STAT1 homodimers bind to identical gamma-activated sequence (GAS) elements, whereas interferon-stimulated gene factor 3 (ISGF3) binds to interferon-stimulated regulatory elements (ISREs).

dimers that are formed in response to many different ligands, including the IFNs, bind to GAS elements whose sequences determine the specificity of the interactions (74). Little current information is available on the regions of STAT dimers that are in contact with the DNA of GAS elements.

Xu et al (75) and Vinkemeier et al (76) have found that the N-terminal domain of STATs 1 and 4 are required for the respective pairs of homodimers to cooperate in binding to tandem GAS sites, which are found in the promoters of some genes that are induced in response to activation of these STATs, for example, the IFN γ gene (75) and the *mig* gene (77). Other genes, for example,

6-16 (78), also have tandem ISREs, and it may be that the cooperative binding of two ISGF3 moieties is required for their optimum expression.

Recent publications have established important connections between STATs 1 and 2 and the CREB-binding protein (CBP)/p300 transcription factors. Zhang et al (79) showed that the N-terminal region of STAT1 interacts with the cyclic-AMP response element binding protein (CREB)-binding domain of CBP/p300 and that the C-terminal region of STAT1 interacts with the domain of CBP/p300, which also binds to the adenovirus protein E1A. Furthermore, both unphosphorylated STAT1 monomers and the phosphorylated STAT1 dimers formed in response to IFN γ are competent to bind to CBP/p300. In transient expression assays, cotransfection of CBP/p300 potentiated and E1A inhibited the activation of a GAS-driven reporter in response to IFN γ . Similarly, Horvai et al (80) showed that the STAT1 and AP1/ets factors that are activated by *Ras*-dependent signaling compete for the limiting amounts of CBP/p300 that each requires for activity. Impressively, microinjection of antibodies directed against CBP/p300 blocks transcriptional responses to IFN γ . Furthermore, Rutherford et al (81) found that the Ets-1 protein of mice binds to an ISRE and may negatively regulate activation by ISGF3. It remains to be seen whether CBP/p300 is required for the transcriptional response to STAT1 homodimers formed in response to IFN α/β , but Bhattacharya et al (82) have shown that CBP/p300 also binds to the C-terminal region of STAT2 and that blockade of this interaction by the adenovirus E1A protein inhibits ISRE-mediated responses to IFN α/β . These fascinating studies provide the first indication of how STATs may interact with the transcriptional machinery.

The mitogen-activated protein kinase (MAPK) cascade is activated by IFN α/β , and the effect of this activation on signaling has been explored. David et al (35) found that ERK2 (the 42-kDa MAPK) binds to a glutathione S-transferase (GST) fusion protein containing the membrane-proximal 50 residues of the cytoplasmic domain of IFNAR1 but not to the full-length cytoplasmic domain of ~ 100 residues. However, there was association between ERK2 and full-length IFNAR1 in vivo. Treatment of cells with IFN β induced the tyrosine phosphorylation and activation of ERK2 and caused it to associate with STAT1, as judged by coprecipitation. Furthermore, expression of a dominant negative form of MAPK inhibited IFN β -induced transcription in a transient cotransfection assay employing an ISRE-driven reporter. It is tempting to connect these observations with those of Wen et al (34), who showed that serine 727 of STAT1, which lies in a MAPK consensus site, is phosphorylated in response to IFN γ and that this phosphorylation increases the response of a GAS-driven promoter to IFN γ . Unfortunately, the connection is not clear. It has not yet been shown that STAT1 is phosphorylated on serine in response to IFN α/β . Wen et al (34) have argued that such phosphorylation is unlikely

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to be important for the activation of ISRE-driven genes because STAT1 β , an alternatively spliced form of STAT1 lacking serine 727, can form ISGF3 and drive the expression of such genes, albeit not as well as STAT1 α (83). This is presumably because STAT2 provides a potent transactivation domain. STAT2 does not contain a serine residue in a MAPK consensus site and is not known to be phosphorylated in response to IFN. Thus, the basis of the cross talk between the IFN α/β and MAPK pathways requires further clarification. In more recent work, Stancato et al (84) showed that Raf1, which lies between Ras and ERK2 in the MAPK cascade, is activated by IFN β in a manner that does not require Ras but does require JAK1. Furthermore, Raf1 activated by IFN β can be coprecipitated with either JAK1 or Tyk2.

IFN α/β treatment causes phosphorylation and activation of cytosolic phospholipase A₂ (CPLA₂), which requires JAK1. Furthermore, JAK1 and CPLA₂ can be coprecipitated (85). Inhibitors of CPLA₂ inhibit IFN α/β -induced expression of ISRE-driven genes, but not of a GAS-driven gene, which implies that CPLA₂ is somehow required for the formation of ISGF3 but not of STAT1 homodimer. The basis for this interesting effect remains to be discovered.

The insulin receptor substrate-1 (IRS1) is phosphorylated on tyrosine residues in response to IFN α/β and, in this state, plays its usual role in bringing phosphatidylinositol 3'-kinase to receptors for activation by tyrosine phosphorylation, by engaging the SH2 domains of the p85 regulatory subunit (86). Burfoot et al (87) found that this activation of IRS1 depends on both JAK1 and Tyk2, thus requiring full function of the IFN α/β receptor. However, the physiological significance of this activation is unclear, because cells lacking both IRS1 and IRS2 show little difference from cells that express these proteins in cell growth inhibition in response to IFN α/β or - γ (87). More experiments need to be done to test the requirement for phosphatidylinositol 3'-kinase in a variety of IFN responses.

Interferon β : A Subtype-Specific Pathway

Humans have at least 12 functional IFN α s, a single antigenically distinct IFN β , and a related IFN ω (88), whereas other species have multiple IFN β subtypes. The human IFN α s are synthesized predominantly by a subset of lymphocytes, and IFN β is made by fibroblasts. Work with different IFN α subtypes, produced by recombinant DNA technology and by purification of natural leukocyte IFNs, has revealed substantial differences in their specific antiviral activities, in the ratios of antiviral to antiproliferative activities, and in a number of additional functions (89-92). A priori, it might be expected that different cell types might respond differentially to the different IFNs, but to date there is no clear evidence of this (for example, see Reference 91). The various IFN α/β s appear to interact with the same receptor and to have antiviral, antiproliferative, and immunomodulatory activities in a number of cell types. The functional significance of the

multiple species, and how functional differences are mediated through apparently identical receptors, remain intriguing questions in this area of research. Interesting differences are emerging, both with respect to IFN-receptor interactions and the induced mRNAs. Mutant cells in the U1 complementation group, lacking Tyk2, are completely defective in response to a purified mixture of natural IFN α s or to recombinant IFN α 1 or α 2; nevertheless, they retain partial responses to IFN β (56) and IFN α 8 (91). How the residual IFN β response is mediated is not yet known, but, importantly, it is not seen in mutant cell lines lacking JAK1, STAT1, or STAT2. It is likely, therefore, to be mediated through JAK-STAT pathways but without an absolute requirement for Tyk2 in the receptor complex. Consistent with this model, IFN β engages the receptor in a distinct fashion. The groups of Revel and Colamonici first noted the rapid, transient tyrosine phosphorylation of a receptor-associated 100-kDa protein in response to IFN β but not to IFN α (93, 94). This protein has recently been identified as the IFNAR2c subunit of the receptor (95, 96). Importantly, IFNAR2c is phosphorylated apparently equivalently in response to IFN α or β , the difference lying in its ability to coprecipitate with IFNAR1 from the complex with IFN β but not IFN α (95, 96). It remains to be established whether this intriguing finding reflects a tighter association of the two subunits in the β versus the α complex, which might in turn reflect structural differences that, if transmitted through the membrane to the intracytoplasmic domains, mediate a differential response. Importantly, β R1, a gene transcribed preferentially in response to IFN β , has been discovered (97). The isolation and characterization of the corresponding promoter should, through the identification of known and novel motifs, provide evidence for the involvement of known signaling pathways and experimental handles to investigate unknown ones.

Modulation of IFN Responses

Proteins of the IRF family, such as IRF2 (98), ICSBP (99), and ICSAT (100), bind to ISREs and negatively regulate expression of the associated genes. These repressors may help to prevent the expression of ISGs in the absence of IFN, to down-regulate the induced response, or both. Treatment of cells with IFN in the presence of protein synthesis inhibitors prolongs ISG transcription (101, 102), indicating that some IFN-induced proteins may help to shut off the response. These may be repressors or other types of inhibitors (see below). A mutant cell line with IFN-independent constitutive expression of ISGs has been isolated. There is little or no defect in shutting off the response to IFN α/β in these cells, suggesting that these two aspects of negative regulation can be distinguished (DW Leaman, A Salvekar, R Patel, GC Sen & GR Stark, unpublished data).

The amount of active STAT1 can be reduced by dephosphorylation (103, 104). Proteasome-mediated degradation may also have a role (105), though this aspect

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is controversial (104). Inhibition of phosphatases by potent agents such as peroxyvanadate stabilizes the ligand-induced phosphorylation of STATs (106, 107) and can also lead, more slowly, to the ligand-independent accumulation of phosphorylated STATs (107). The inhibited phosphatases may operate on phosphorylated STATs in the nucleus or on phosphorylated JAKs or receptor subunits at the plasma membrane (103, 106). Phosphatases with SH2 domains are especially good candidates for the latter function, and SHP-1 has been implicated in this function in hematopoietic cells (108, 109). Other phosphatases would have to assume this role in most other cell types, where SHP-1 is not expressed. Phorbol esters, which inhibit signaling in response to $IFN\alpha/\beta$, can do so by activating one or more tyrosine phosphatases that selectively dephosphorylate Tyk2 but not JAK1 or IFNAR1 (110). Decreased availability of p48 may also play a role (111). The ISGF3-mediated response to $IFN\alpha/\beta$ is initiated by the rapid formation of ISGF3, but at least for some genes, it is likely to be sustained by IRF1 (69). In human fibroblasts, the level of ISGF3 declines over the course of a few hours and returns to a basal level after 4 h. However, at this time, the transcription rate of the 6-16 gene is still at a maximum, coincident with the maximum in the IFN-induced expression of IRF1 (69). The eventual return of 6-16 transcription to a basal level, in about 8 h, corresponds to the decline of IRF1. By studying mice null for expression of p48, IRF1, or both, Kimura et al (112) have shown that p48 and IRF1 do not have redundant functions but instead complement each other in the responses to both $IFN\alpha/\beta$ and $IFN\gamma$.

FUNCTIONS INDUCED BY INTERFERONS

Antiviral Activities

The ability of IFNs to confer an antiviral state on cells is their defining activity as well as the fundamental property that allowed their discovery (113). IFNs are essential for the survival of higher vertebrates because they provide an early line of defense against viral infections—hours to days before immune responses. This vital role has been demonstrated by the exquisite sensitivity to virus infections of mice lacking both $IFN\alpha/\beta$ and γ receptors (114). Multiple, redundant pathways have evolved to combat different types of viruses and the various compensatory defense mechanisms that different viruses have evolved (see below). Any stage in virus replication appears to be fair game for inhibition by IFNs (115), including entry and/or uncoating [simian virus 40 (SV40), retroviruses], transcription [influenza virus, vesicular stomatitis virus (VSV)], RNA stability (picornaviruses), initiation of translation (reoviruses, adenovirus, vaccinia), maturation, and assembly and release (retroviruses, VSV).

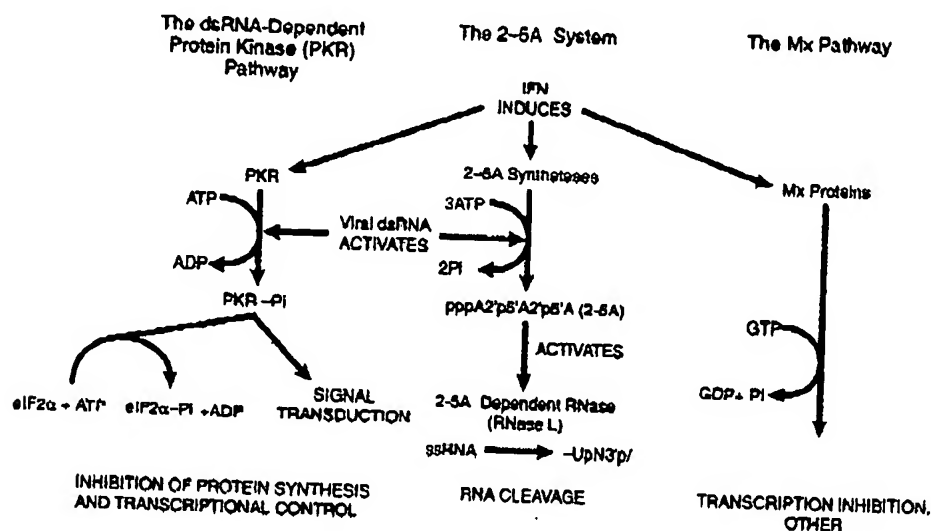


Figure 5 Antiviral mechanisms of interferon (IFN) action.

PKR The best-characterized IFN-induced antiviral pathways utilize the dsRNA-dependent protein kinase (PKR), the 2-5A system, and the Mx proteins (Figure 5). PKR is a serine-threonine kinase with multiple functions in control of transcription and translation (116, 117). PKR is normally inactive, but on binding to dsRNA, it undergoes autophosphorylation and subsequent dsRNA-independent phosphorylation of substrates. Two conserved dsRNA-binding motifs are present in the N-terminal regulatory half of PKR. The first mediates dsRNA-binding activity and includes residues critical for binding to dsRNA, highly conserved within a large family of dsRNA-binding proteins. No RNA sequence specificity is required for dsRNA to bind to PKR. The resulting conformational change in the enzyme probably unmasks its C-terminal catalytic domain (118). The antiviral effect of PKR is due to its phosphorylation of the alpha subunit of initiation factor eIF2. This phosphorylation results in the formation of an inactive complex that involves eIF2-GDP and the recycling factor eIF2B, resulting in rapid inhibition of translation. Apoptosis may also play a role in the antiviral effect of PKR (see below). Overexpression of PKR leads to the suppression of encephalomyocarditis virus (EMCV) replication in cultured cells (119). In addition, a dominant negative PKR mutant or an antisense PKR cDNA construct suppresses the anti-EMCV effect of IFNα and IFNγ in promonocytic U-937 cells (120). Poly(I):poly(C) or IFNγ treatment extends the survival of wild-type but not PKR-null mice after infection with EMCV (121). In contrast, redundancy in the antiviral pathways is apparent because IFNα extends to the same extent the survival of wild-type and PKR-null mice after EMCV infection.

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THE 2-5A SYSTEM This system is a multienzyme pathway (Figure 5) in which IFN-inducible 2-5A synthetases are stimulated by dsRNAs, often of viral origin, to produce a series of short, 2',5'-oligoadenylates (2-5A) that activate the 2-5A-dependent RNase L (122, 123). Activation of this pathway leads to extensive cleavage of single-stranded RNA (124–126). The 2-5A synthetases (40, 46, 67, 69, 71, and 100 kDa) are encoded by multiple genes and reside in different parts of the cell (127–130). 2-5A binds to inactive, monomeric RNase L, inducing the formation of the homodimeric, active enzyme (131–133). The activation of RNase L is reversible (134). Its N-terminal half is a repressor that contains a repeated P-loop motif and nine ankyrin repeats, both involved in 2-5A binding. The C-terminal half contains a region of protein kinase homology, a cysteine-rich domain, and the ribonuclease domain (135, 136). The isolated C-terminal half of RNase L cleaves RNA in the absence of 2-5A (137). There are striking similarities between RNase L and IRE1p, a yeast endoribonuclease that functions in Hac1 mRNA splicing in the unfolded protein response (138). An intriguing possibility is that RNase L might exist as a member of a family of regulated nucleases with diverse functions in different organisms.

The functions of the 2-5A system have been explored through genetic manipulation of RNase L. Cells expressing a dominant negative derivative were defective in expressing the anti-EMCV and antiproliferative activities of IFN α/β , whereas overexpression of RNase L blocked vaccinia virus and HIV-1 replication (136, 139; RK Maitra & RH Silverman, unpublished data). RNase L-null mice are deficient in both the anti-EMCV effect of IFN α and in several apoptotic pathways (140). Although IFN α treatment extended the survival of both wild-type and RNase L-null mice after EMCV infection, the RNase L-null mice died several days earlier, lending support to cell culture studies linking the 2-5A system to the anti-EMCV effect of IFN (123). The ability of RNase L to be activated by small molecules opens up possibilities for drug design and development. In one such example, RNase L was recruited by 2-5A-antisense oligonucleotides to cleave respiratory syncytial virus M2 RNA selectively, thus blocking viral replication in human tracheal epithelial cells (141, 142). A mammalian 2-5A system has also been cloned in transgenic tobacco plants, resulting in resistance to several different viruses (143, 144).

THE MX PROTEINS Mx proteins are IFN-inducible, high-abundance 70- to 80-kDa GTPases in the dynamin superfamily (145, 146). Mx proteins and dynamins self-assemble into horseshoe- and ring-shaped helices and other helical structures (147–149). Human MxA forms tight oligomeric complexes in cell-free systems and in intact cells (150, 151). The Mx proteins interfere with viral replication, impairing the growth of influenza and other negative-strand RNA viruses at the level of viral transcription and at other steps. The

murine nuclear protein Mx1 suppresses the growth of influenza, Thogoto, and tick-born Dhori viruses, and the human cytoplasmic protein MxA inhibits the growth of influenza, VSV, measles, Thogoto, bunya, phlebo, hanta, and human parainfluenza 3, but not Dhori viruses (152-157). Mutant forms of Mx proteins lacking the ability to bind or hydrolyze GTP fail to suppress viral replication. However, the binding, not the hydrolysis, of GTP is required to inhibit VSV transcription by MxA in vitro (158, 159). Mx proteins are believed to interfere with the trafficking or activity of viral polymerases (160). Furthermore, MxA specifically binds to Thogoto-virus ribonucleoprotein complex (O Haller, personal communication). Murine Mx1 inhibits the primary transcription of influenza virus, whereas human MxA acts in the cytoplasm to inhibit a later step in the viral life cycle (161). Although PKR and Mx genes are induced preferentially by type I IFNs, 2-5A-synthetase and RNase L are induced by both types (116, 135, 162, 163). Also, the induction by IFN γ (but not IFN α/β) of nitric oxide synthase in mouse macrophages inhibits the growth of ectromelia, vaccinia, and HSV-1 viruses (164). Therefore, different antiviral pathways may be induced in different cell types, depending on the type of IFN involved.

Many IFN-induced proteins are poorly characterized, and some of these are very likely to possess antiviral activity. For instance, expression of the IFN-inducible 9-27 protein led to a partial inhibition of VSV replication (165). Clearly, the enormous selective pressures imposed by viruses have resulted in a rich and diverse set of antiviral pathways.

VIRAL INHIBITION OF THE IFN RESPONSE Hardly surprising, viruses fight back, not only against host defenses in general (166-169) but also against the IFN systems in particular, both through novel mechanisms and by subverting host systems through the synthesis of novel proteins and proteins that mimic and thus interfere with host proteins (e.g. the IFN receptors; see References 169, 170). There is evidence for the inhibition of the 2-5A-dependent RNase L in response to EMC infection (171) and for a cellular protein inhibitor of RNase L (172), but the most extensively studied examples involve the inhibition of PKR. At least four different mechanisms are used, including inhibitory viral RNA, inhibitory viral or cellular proteins, and proteolytic cleavage. Best studied is the adenovirus virus-associated (VA) RNA, which binds to but does not activate PKR (173). An important fact is that mutant viruses lacking VA RNA are more sensitive to IFN-mediated inhibition (174). Epstein-Barr virus-encoded small nonpolyadenylated RNAs (EBER) may perform a similar function for Epstein-Barr (EB) virus (175), although an EBER-negative strain shows no obviously enhanced sensitivity to IFNs in vitro (176). Examples of proteins that sequester the viral dsRNA activators of PKR are the reovirus sigma 3 capsid protein (177)

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and the vaccinia virus E3L protein (178). The HIV transcriptional transactivator (TAT) (179, 180) and hepatitis C virus NS5A (181) proteins appear to inhibit by interacting with PKR directly. In response to EMC infection, proteolytic cleavage of PKR in poliovirus-infected cells and sequestration of the enzyme occur (182, 183). Particularly interesting are the cellular protein systems that inhibit PKR in response to influenza virus infection. p58(IPK), a cellular protein inhibitor of PKR, is inhibited by I-p58(IPK), which is apparently inactivated in response to infection. I-P58(IPK) has recently been identified as the molecular chaperone hsp40; this identification revealed that the influenza virus regulates PKR activity by recruiting a cellular stress protein (184). Both cells and viruses have developed elegant mechanisms to control PKR, which shows the importance of this enzyme in controlling cellular functions (see below) and virus replication. As a variant on this theme, SV40 can restore efficient translation in cells, despite the elevated levels of phosphorylated eIF2 α that result from activating PKR, because the translational rescue mediated by the SV40 large T antigen occurs downstream of the phosphorylation of eIF2 α (185).

Cell death in response to virus infection may be mediated by apoptosis as well as necrosis (186). Interestingly, cells from mice lacking the 2-5A-dependent RNase L or PKR show defects in apoptosis (140, 187), consistent with a possible role for these enzymes in virus-induced, IFN-mediated cell death. Poxviruses produce CrmA, and the Kaposi's sarcoma herpes virus produces FLIPs (inhibitors of the apoptotic ICE and FLICE proteases, respectively), presumably to suppress host-cell suicide and inflammatory responses (188, 189). Other viral anti-apoptotic genes resemble the mammalian bcl-2 gene, which suppresses apoptosis (186, 190).

The adenovirus E1A and human papilloma virus E6 and E7 proteins inhibit the production and action of IFN at the level of transcription (66, 191-194). For E1A, the effect on the transcription of ISGs is mediated, in part at least, through a reduction in functional p48 (192, 193) and probably also by sequestering p300/CBP, required for transcriptional activation through STAT2 (82). A similar inhibition at a transcriptional level of IFN production and action is mediated by the Kaposi's sarcoma herpes virus through the production of an inhibitory mimic of IRF (169). In additional strategies, the poxviruses produce soluble IFN α and IFN γ receptors (116), and the EB virus generates an IL-10 analog. Interestingly, the type I IFN receptor mimic produced by the vaccinia virus shows a wide species specificity, consistent with the broad host range of the virus (195). The IL-10 analog probably performs a dual function for the virus, inhibiting the production of IFN γ and activating the B lymphocytes necessary for virus replication (196).

The IFN systems are subject to cellular control during development and differentiation and are subject to inhibition by viruses. The multiple mechanisms

involved emphasize the importance of these systems to both cells and viruses. Conversely, the IFN systems are not, of course, the only host defense systems against which viruses retaliate. Indeed, to know which host defenses are important, viruses must be investigated.

Inhibition of Cell Growth

IFNs inhibit cell growth and control apoptosis, activities that affect the suppression of cancer and infection. Genes have been identified that are important for the apoptotic, but not the growth inhibitory, effects of IFN γ (197). Therefore, these two activities of IFNs are considered separate but related topics.

Different cells in culture exhibit varying degrees of sensitivity to the antiproliferative activity of IFNs. In some cases, growth arrest may be due to differentiation, particularly when IFNs are used in combination with other agents such as retinoids (198,199). Specific IFN-induced gene products have not been linked directly to antiproliferative activity. However, IFN α has been shown to target specific components of the cell-cycle control apparatus, including c-myc, pRB, cyclin D3, and cdc25A (200–203). Lymphoblastoid Daudi cells are exquisitely sensitive to the antiproliferative effects of IFN α , which lead to a rapid shutdown of c-myc transcription, possibly through a decrease in the activity of the transcription factor E2F (202). Cells expressing a transdominant mutant of PKR fail to suppress c-myc in response to IFN, although the phosphorylation of pRB is suppressed (204). PKR may play a subtle role in cell growth regulation. Early-passage embryo fibroblasts (MEFs) established from PKR-null mice achieve saturation densities similar to those of wild-type MEFs, whereas PKR-null cells consistently achieve higher saturation densities beyond five passages. The doubling times of wild-type and PKR-null cells do not differ appreciably between early and late passages, however (S Der & BRG Williams, unpublished data). This phenotype is similar to the increased saturation densities described for MEFs derived from p53-null or p21/WAF1-null mice (205) and may result from increased resistance to apoptosis, induced by growth-factor deprivation in the absence of PKR.

The phosphorylation of pRB by IFN is suppressed by the inhibition of cdk4 and cdk6. This inhibition is achieved through the suppression of cyclin D3 and by preventing the activation of cdk2-cyclinA and cdk-cyclinE, thereby inhibiting the phosphatase cdc25A (203). This mechanism of growth suppression is distinct from that of other growth-inhibitory cytokines such as TGF β because it does not appear to involve the induction of cdk inhibitors such as p21, p27, and p57^{kip}. Cell-type differences clearly complicate any mechanistic understanding of the antiproliferative effects of IFN. For example, in contrast to the Daudi cells discussed above, the induction of p21-cdk2 has been correlated with growth inhibition of the prostate cancer cell line DU145 by IFN α (206).

Control of Apoptosis

IFNs are essential for host responses to viruses and some other microbial pathogens, events that often culminate in apoptosis. However, development in the mouse proceeds normally in the absence of functional IFN α/β and IFN γ receptors (114). IFNs have either pro- or anti-apoptotic activities, depending on factors such as the state of cell differentiation. For instance, IFN γ either induced or inhibited the apoptosis of murine pre-B cells or B chronic lymphocytic leukemia cells, respectively (207–209). Similarly, IFN γ promoted either proliferation or apoptosis in malignant human T cells, depending on the presence or absence of serum and the levels of the IFN γ receptor (210). The involvement of IFNs in apoptosis is interwoven with the roles of other modulators of apoptosis and the enzymes they regulate. For example, dsRNA produced during viral infections, and lipopolysaccharide (LPS), an endotoxin of gram-negative bacteria, are potent inducers of apoptosis. Interestingly, either dsRNA or LPS induces the synthesis of IFNs. The induction of IFN γ by LPS requires the activity of caspase-1 (ICE) to process the IFN γ -inducing factor IGIF or IL-18 (211, 212). Similarly, dsRNA induces the production of IFN α/β and is a pro-apoptotic agent (187).

Investigations into molecular pathways mediating IFN-induced apoptosis have focused either on the antiviral enzymes (see below) or on identifying other proteins in the pathway. Several genes have been cloned that, when down-regulated, suppress the growth inhibitory or apoptotic activities of IFN γ in HeLa cells. Five novel genes for death-associated proteins, DAP-1 to -5, and two other proteins (thioredoxin and the protease cathepsin D) play a role in these processes (197, 213–218). Down-regulation of DAP-1 (a proline-rich protein), DAP-2 (DAP kinase), DAP-3, or cathepsin D with antisense RNAs blocked the apoptotic activity but not the cytostatic effect of IFN γ . DAP-2, a calmodulin-dependent protein kinase with a consensus death domain, localizes to the cytoskeleton, and its expression is lost frequently in human tumors (218, 219). DAP-2 is a tumor suppressor gene that couples the control of apoptosis to metastasis (219a). DAP-5 was identified from a partial cDNA encoding a dominant negative protein related to the protein-synthesis initiation factor eIF4G (217). It will be interesting to determine how all of these proteins interact with each other and with other intracellular apoptotic factors.

Effects of IFNs on the Immune System

The immunomodulatory actions of IFNs have been studied extensively, but because of space limitation, they cannot be discussed in detail here. Several reviews on IFN α/β and IFN γ biology have been published recently, and the reader is referred to these for more details (12, 115, 220, 221). Here we identify major recent advances in understanding the roles of IFNs in promoting

immune responses, and we provide examples of how the actions of $\text{IFN}\alpha/\beta$ and $\text{IFN}\gamma$ diverge. IFNs are known to profoundly affect nearly all phases of innate and adaptive immune responses. Within the IFN family, $\text{IFN}\gamma$ plays the predominant immunomodulatory role. It is produced by a restricted set of immune cells (T cells and natural killer cells) in response to immune and/or inflammatory stimuli and functions to stimulate the development and actions of immune effector cells. The immunomodulatory actions of $\text{IFN}\alpha/\beta$ are more restricted: They are directed largely at promoting responses that provide the host with adaptive immune response mechanisms to resist viral infection.

IFN, ANTIGEN PROCESSING AND PRESENTATION, AND DEVELOPMENT OF CD8^+ T-CELL RESPONSES One unarguable role of IFNs in promoting protective immune responses is their ability to regulate the expression of proteins encoded in the major histocompatibility complex (MHC). All IFN family members share the ability to enhance the expression of MHC class I proteins and thereby to promote the development of CD8^+ T-cell responses (221). This expression is known to be driven by IRF1, the transcription factor predominantly responsible for activating MHC class I gene transcription (222, 223). Cells from mice with targeted mutations in either the $\text{IFN}\gamma$ or $\text{IFN}\alpha/\beta$ receptor systems, STAT1, PKR, or IRF1 fail to up-regulate MHC class I proteins on their surface in response to stimulation by the appropriate IFN. In contrast, $\text{IFN}\gamma$ is uniquely capable of inducing the expression of MHC class II proteins on cells, thereby promoting enhanced CD4^+ T-cell responses (221, 224). This response depends on a distinct transactivating factor, CIITA. Cells from human patients with the rare abnormality bare-lymphocyte syndrome, characterized by the absence of CIITA, fail to express MHC class II proteins either constitutively or following exposure to $\text{IFN}\gamma$. $\text{IFN}\gamma$ induces MHC class II protein expression in a wide variety of different cell types, such as mononuclear phagocytes, endothelial cells, and epithelial cells, but it inhibits IL-4-dependent class II expression on B cells (225). The molecular basis for this discordant effect is unknown.

IFNs also play an important role in antigen processing by regulating the expression of many proteins required to generate antigenic peptides. $\text{IFN}\gamma$ modifies the activity of proteasomes by modulating the expression of both enzymatic and nonenzymatic components (221, 226). The proteasome is a multisubunit enzyme complex that is responsible for the generation of all peptides that bind to MHC class I proteins. In unstimulated cells, it contains three enzymatic subunits: α , β , and γ . However, following treatment of cells with $\text{IFN}\gamma$, transcription of the α , β , and γ genes decreases, and transcription of three additional genes encoding different enzymatic proteasome subunits, LMP2, LMP7, and MECL1, increases. This leads to the formation of different proteasomes

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containing these subunits and possessing a different substrate specificity, thereby altering the types of peptides produced and eventually presented to the immune system. IFN γ also induces the expression of a nonenzymatic proteasome subunit, PA28 (also known as the 11S regulator), which binds to proteasome enzyme components and alters their specificity (227, 228). Finally, IFN γ increases the expression of TAP1 and TAP2, which transfer peptides generated by the proteasome in the cytoplasm into the endoplasmic reticulum, where they bind to nascent MHC class I chains (229, 230). Thus, IFNs enhance immunogenicity by increasing the quantity and repertoire of peptides displayed in association with MHC class I proteins.

IFN γ AND DEVELOPMENT OF THE CD4⁺ HELPER T-CELL PHENOTYPE Activated human and murine CD4⁺ T cells can differentiate into two polarized subsets, defined by the cytokines they produce when stimulated (231). In mice, T helper 1 (Th1) cells have the selective ability to synthesize IFN γ , lymphotoxin (LT), and IL-2 and to promote cell-mediated immunity and delayed type hypersensitivity (DTH) responses. In contrast, murine Th2 cells selectively produce IL-4, IL-5, IL-6, and IL-10 and thereby facilitate antibody production and the development of humoral immune responses. IFN γ has an important effect on Th1 cell development. In vitro, antibody-mediated neutralization of IFN γ greatly reduces the development of Th1 cells and augments the development of Th2 cells (232). Similar effects are seen in mice lacking the ability to respond to IFN γ , i.e. STAT1-null mice. However, administration of exogenous IFN γ , in vitro or in vivo, does not drive a Th1 response. Thus, IFN γ is necessary but not sufficient for Th1 development.

IFN γ plays a dual role in this process. First, it facilitates Th1 production by enhancing the synthesis of IL-12 in antigen-presenting cells (233–235). IL-12 is the proximal effector that drives developing CD4⁺ T cells to become Th1 cells (232, 236). In addition, IFN γ maintains expression of the β 2 subunit of the IL-12 receptor on developing CD4⁺ T cells, thereby preserving their capacity to respond to IL-12 (237). Second, IFN γ blocks the development of Th2 cells by inhibiting the production of IL-4, which is required for Th2 formation (238), and by preventing Th2-cell proliferation (239). Th1 cells are not affected in this manner because they become insensitive to IFN γ as a result of IFN γ -dependent down-regulation of the expression of IFNGR2 (36, 37).

IFN, MACROPHAGE ACTIVATION, AND CELLULAR IMMUNITY Macrophages function as a key effector cell population in innate and adaptive immune responses. To carry out these functions, they must first become activated, a process involving a reversible series of biochemical and functional alterations that provide them with enhanced cytotoxic activities (240). Through the use

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of neutralizing IFN γ -specific monoclonal antibodies and gene-targeted mice, it has been possible to establish unequivocally the predominant role played by IFN γ in generating activated macrophages, both in vitro and in vivo (241–243). Importantly, the macrophage-activating activity of IFN γ is not provided by IFN α/β . Supporting data come from studies demonstrating that IFN γ -unresponsive mice or humans (i.e. IFN γ -null, IFN γ receptor-null, or STAT1-null mice or patients with inactivating mutations in the IFNGR1 gene) are highly susceptible to infection with a variety of microbial pathogens such as *Listeria monocytogenes*, *Toxoplasma gondii*, *Leishmania major*, and several different strains of *Mycobacteria* (41, 242–245). Increased susceptibility to infection occurs in IFN γ -unresponsive hosts despite their ability to maintain an unaltered capacity to produce and respond to IFN α/β .

Activated macrophages use a variety of IFN γ -induced mechanisms to kill microbial targets. Two of the most important involve the production of reactive oxygen and reactive nitrogen intermediates. Reactive oxygen intermediates are generated as a result of the IFN γ -induced assembly of NADPH oxidase, formed as a result of the induced translocation of two cytosolic enzyme subunits to the plasma membrane, where they combine with a membrane-associated electron transport chain component, cytochrome *b558* (246). This enzyme effects a one-electron transfer to oxygen, producing superoxide anion, which, in turn, is used to generate additional toxic oxygen compounds such as hydrogen peroxide, hydroxyl radicals, and singlet oxygen. Reactive nitrogen intermediates, particularly nitric oxide (NO), are generated in murine macrophages as a result of the IFN γ -dependent transcription of the gene encoding the inducible form of nitric oxide synthase (iNOS), which catalyzes the formation of large amounts of NO (247). NO is thought to kill target cells by one of two mechanisms. First, it can form an iron-nitrosyl complex with the Fe-S groups of aconitase, complex I and complex II, thereby inactivating the mitochondrial electron transport chain. Alternatively, NO can react with superoxide anion to form peroxynitrite, which decays rapidly to form highly toxic hydroxyl radicals. Although iNOS is induced in murine macrophages in an IFN γ -dependent manner, it is not induced in human mononuclear phagocytes exposed to the same stimuli. The molecular basis for this difference has not yet been defined.

IFN AND HUMORAL IMMUNITY IFNs play complex and sometimes conflicting roles in regulating humoral immunity. Most analyses have attempted to define the influence of IFN γ in the process, although more recent observations suggest that IFN α/β may also induce many of the same biological effects. IFNs exert their effects either indirectly (as described above), by regulating the development of specific T helper cell subsets, or directly at the level of B cells. In the latter case, IFNs are predominantly responsible for regulating three

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specialized B-cell functions: development and proliferation, immunoglobulin (Ig) secretion, and Ig heavy-chain switching.

The best-characterized action of IFNs directed toward B cells is their ability to influence Ig heavy-chain switching. Ig class switching is significant because the different Ig isotypes promote distinct effector functions in the host. By favoring the production of certain Ig isotypes while inhibiting the production of others, IFNs can facilitate interactions between the humoral and cellular effector limbs of the immune response and increase the host defense against certain bacteria and viruses. In vitro, IFN γ is able to direct immunoglobulin class switching from IgM to the IgG2a subtype in LPS-stimulated murine B cells (248) and to IgG2a and IgG3 in murine B cells that have been stimulated with activated T cells (249). Moreover, IFN γ blocks IL-4-induced Ig class switching in murine B cells from IgM to IgG1 or IgE (250). The validity of these observations has been tested stringently by injecting mice with polyclonal anti-IgD serum, a polyclonal activator of B cells. These mice produced large quantities of IgG1 and IgE. However, when IFN γ was administered prior to anti-IgD treatment, the mice produced high levels of IgG2a and decreased levels of IgG1. Thus, IFN γ is clearly an important regulator of Ig class switching in vivo.

A role for type I IFNs in this process has also been identified (251). Of particular importance are experiments using mice that lack receptors for IFN γ , IFN α/β , or both (114). The mice were infected with lymphocytic choriomeningitis virus (LCMV), and the profiles of the LCMV-specific antibodies generated were determined. Comparable levels of LCMV-specific IgG2a antibodies were observed in the sera of normal mice and of mice unresponsive to either IFN γ or IFN α/β . In contrast, IgG2a antibodies were not produced in mice lacking responses to both types of IFN. These results demonstrate that if induced during the immune response, IFNs α/β can indeed function in a manner redundant to IFN γ in effecting Ig class switching.

ADDITIONAL FUNCTIONS OF PROTEINS INVOLVED IN IFN RESPONSES

JAKs

JAKs can auto- and transphosphorylate, and it is reasonable to assume that they phosphorylate the receptors and the proteins recruited to them, foremost among which are STATs. The interaction of SH2 domains with receptor subunit phosphotyrosine motifs clearly plays a major role in recruitment. But it is increasingly unlikely that this is the whole story, and recruitment directly by JAKs is an interesting alternative. For example, it appears possible that signaling by growth hormone can be achieved with a receptor entirely lacking phosphotyrosine

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motifs (252). Fujitani et al (253) have presented evidence for the recruitment of STAT 5 through JAK2, and a number of additional JAK-signaling component interactions have been reported (see below). More generally, JAK1 and JAK2 are present in the cell nucleus as well as in the cytoplasm and at membranes (254; A Ziemiecki, personal communication). Initial results with a dominant negative derivative of JAK1 raise the possibility of a constitutive requirement for JAKs early in zebra fish development (255). JAK3-null mice show no obvious defect in early development (256, 257). JAK1-null mice, however, are runted, fail to nurse, and die perinatally. They also appear to have a sensory neuron defect, which includes a failure of explanted dorsal root ganglion neurons to survive when cultured in the presence of IL-6, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), or cardiotrophin 1 (CT-1), all neurotrophic factors that signal to the JAKs through the IL-6 receptor family (S Rodig & R Schreiber, unpublished data). JAK2-null mice die early in embryogenesis, consistent with a failure of hematopoiesis (E Parganas & JN Ihle, personal communication). Of course, it remains to be established that any of the defects in the knockout mice reflect a requirement for additional JAK functions. That said, it has become increasingly clear that just as JAKs may not be the only mediators of STAT activation, STATs are not the only targets of activated JAKs. Evidence for this comes from both protein association data and functional experiments. For example, growth hormone, IL-11, and OSM all promote the association of JAK2 with Shc and Grb2 (258-260). Work with a JAK2-null cell line has established that the phosphorylation of Shc in response to growth hormone depends on JAK2 (261), consistent with a requirement for cotransfected JAK2 to achieve MAP kinase activation in response to activation of a transfected growth hormone receptor (262). Raf1 associates with JAK2 when coexpressed in the baculovirus system and in erythropoietin- or IFN-treated cells (262). Therefore, JAK-dependent MAP kinase activation by different cytokines or growth factors may occur through recruitment to the JAKs as well as through the well-established pathway involving receptor tyrosine motifs (262). Additional proteins reported to interact with JAKs include SHP1 and SHP2, Vav, Fyn, Btk, Tec, and c-Abl (108, 109, 263-268). For c-Abl, constitutive JAK activation correlated with transformation and was lost on inactivation of a temperature-sensitive c-Abl protein (267). Early suggestive evidence for an additional role for JAKs came from the demonstration that JAK1 is activated in response to EGF but is not required for STAT activation (269). The function of activated JAK1 in this response remains to be established. Similarly, the work of many groups on truncated cytokine receptors has established a requirement for the juxtamembrane Box1 and Box2 motifs and for JAK (but not STAT) activation to stimulate a mitogenic response through pathways yet to be defined (270, 271). IRS1 has been shown to coimmunoprecipitate with

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JAK2, and the ability of the growth hormone receptor to transduce the signal for IRS1 depends on the same region of the receptor required for JAK2 binding (272). More recently, work with JAK-null cell lines has established that the activations of IRS1 and phosphatidylinositol 3' kinase by IL-4, OSM, and IFNs are JAK dependent (87, 273). For IFNs, work with JAK1-null cells has established that JAK1 is required for the activation of cytosolic phospholipase A2 by IFN α (85). Data obtained with a kinase-negative derivative of JAK1 and mutant receptors have raised the possibility that additional JAK-dependent pathways may be required in the antiviral responses to IFN γ and IFN α/β (23, 274). Also, in the most detailed study of the activation of the MAP kinase pathway by IFN α , Lamer et al have concluded that JAK1 is essential for the activation of Raf1 and the ERK/MAP kinases (84). Finally, Sugamura et al have implicated JAKs 2 and 3 in activating the signal transducing adaptor molecule, which is involved in both c-myc induction and cell growth in response to IL-2 and GM-CSF (275).

STATs

Evidence is accumulating that STATs play an important role distinct from their well-known function as inducible transcription factors. Three distinct observations reveal an important role for STAT1 in the constitutive expression of certain genes. The expression of IRF1 is low in STAT1-null U3A cells and becomes significant when STAT1 is expressed from a transgene in U3A-R cells (83). The caspase family members ICE, Cpp32, and Ich-1 are expressed at levels 10- to 15-fold lower in U3A cells than in U3A-R or wild-type cells, leading to substantial defects in response to pro-apoptotic signals (275a). Expression of both LMP2 and LMP7 is almost completely absent in U3A cells and is restored in U3A-R cells (Chatterjee-Kishore et al, unpublished data). The defects in caspase expression were corrected when U3A cells were complemented with the Y701F mutant of STAT1, ruling out the possibility that a STAT-STAT dimer stabilized by SH2-phosphotyrosine interactions can be responsible (275a). A strong conclusion is that STAT1 is required for the constitutive expression of some genes, either alone as monomers or, more likely, in combination with transcription-factor partners still to be identified. It remains to be seen whether other STATs have similar functions. As noted above (see also Reference 75), STAT dimers interact with each other and with several different transcription factors, primarily through the N-terminal domains, and STAT2 also uses its N-terminal domain to bind to the IFNAR2c subunit of the receptor (59). We thus imagine that the N-terminal domain of STAT1 may mediate its binding to transcription-factor partners required for constitutive gene expression.

A recent report (49) reveals a scaffolding role for STAT3, which uses its SH2 domain to bind to the tyrosine-phosphorylated cytoplasmic tail of IFNAR1 in the

activated IFN α/β receptor. STAT3 also binds to phosphatidylinositol 3'-kinase, thus bringing it to the receptor. This binding is followed by phosphorylation of phosphatidylinositol 3'-kinase on tyrosine. The functional consequences of this activation by IFN α/β of an additional signaling pathway remain to be elucidated. That at least some of the STATs can serve alternative functions alerts us to the possibility that STAT-null mice may exhibit phenotypes that do not result solely from the lack of STAT activation in response to cytokines or growth factors.

PKR

The activity of PKR in regulating translation is supplemented by its role as a signal-transducing kinase in pathways activated by dsRNA, LPS, and different cytokines (117, 276). In human and mouse cells, activation of PKR by dsRNA leads to activation of NF κ B through PKR-mediated phosphorylation of I κ B (121, 277-279), and recombinant PKR can activate NF κ B and induce DNA-binding activity in cell lysates (277). It is likely that PKR regulates an I κ B kinase, and two recent publications have now identified such an enzyme, capable of phosphorylating I κ B on the two serine residues appropriate for *in vivo* function (280, 281).

PKR also plays a role in signal transduction by IFN α , IFN γ , dsRNA, TNF α , LPS, and platelet-derived growth factor (PDGF), revealed largely through experiments with MEFs derived from PKR-null mice. IFNs, dsRNA, TNF α , and LPS all fail to activate the DNA-binding activity of IRF1 in PKR-null MEFs, resulting in a selective defect in the induction of genes dependent on IRF1 (or NF κ B) (121, 187, 279). Several genes important in mounting different aspects of host resistance to infection can now be classified as wholly or partially dependent on PKR, including genes involved in antigen presentation (class I MHC), chemotaxis (the chemokines IP-10, myg, JE, and Rantes), antimicrobial activity (iNOS), and apoptosis (FAS). The induction of the cell adhesion molecules VCAM and E-selectin by dsRNA is also mediated through a PKR-dependent pathway (282; S Bandyopadhyay & BRG Williams, unpublished data). Induction of the immunoglobulin κ gene by LPS or IFN γ is mediated by PKR, probably through activation of IRF1 (283).

The mechanisms of activation of PKR by cytokines require further investigation. It is not known whether activation occurs through JAK-dependent pathways or through other signals generated by receptor engagement (for example, Ca²⁺). An interaction of PKR with STAT1 has been reported (284) but does not appear to be functional, because STAT1-dependent activities are unaffected in PKR-null cells (279). In contrast, the induction of c-fos and c-myc expression by PDGF can be blocked by inhibitors of PKR or by an antisense oligonucleotide against PKR mRNA (285). In accord with this, the

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PDGF-induced binding of STAT3 to the GAS element of the c-fos promoter is defective in extracts from PKR-null MEFs compared with extracts from wild-type cells, although the response of STAT3 to other stimuli remains unaffected (A Deb & BRG Williams, unpublished data).

In addition to mediating an important antiviral activity of PKR, the phosphorylation of eIF2 α is involved in antiproliferative activities because of this kinase. The most direct evidence comes from studies of the expression of PKR in *Saccharomyces cerevisiae*, where inducible expression of wild-type but not kinase-inactive PKR results in inhibition of growth, which can be reversed by the coexpression of a mutant yeast eIF2 α that is not phosphorylated by PKR (286, 287). The induction of tumor formation by mutant PKR proteins (288–290) could be due to failure to appropriately regulate eIF2 or to interactions with other cellular proteins involved in cell growth control.

INVOLVEMENT OF PKR IN APOPTOSIS The mechanisms and signaling mediators that regulate virus-induced apoptosis are not well understood, but it has long been recognized that a combination of IFN and dsRNA is cytotoxic. Because PKR inhibits the growth of yeast and mammalian cells, it is an attractive candidate for involvement in the apoptosis mediated by dsRNA. In support of this idea, overexpression of PKR induces apoptosis through a mechanism dependent on Bcl2 and ICE (291, 292). Normal levels of PKR are required to mediate an apoptotic response to different stimuli, including dsRNA. For example, reduction of PKR levels by antisense oligonucleotides in promonocytic U937 cells inhibits the apoptosis induced by TNF α (293). MEFs derived from PKR-null mice resist apoptotic cell death in response to dsRNA, TNF α , or LPS through a mechanism linked to a defect in activating the DNA-binding activity of IRF1 (187). These results reveal an unexpected role for PKR in mediating stress-induced apoptosis through regulation of IRF1 activity.

Apoptosis is also important in T-cell development. Although thymocytes of wild-type mice express relatively high levels of PKR, the size of the thymus and the ratios of peripheral T-cell subsets are normal in PKR-null mice (S Kadererit & BRG Williams, unpublished data). Therefore, PKR expression in thymocytes is not essential for apoptosis associated with negative and positive selection. Fas mRNA expression is strongly induced in wild-type cells by dsRNA, LPS, and IFN γ , but with the exception of IFN γ , the induction is much reduced in MEFs derived from PKR-null mice (187). Death signals transduced by the Fas receptor depend on the presentation of the ligand Fas L and are largely restricted to a few cell types, such as activated cytotoxic T cells. However, the induction of Fas on wild-type MEFs by dsRNA results in sensitization of the cells to killing by an anti-Fas antibody that stimulates the Fas receptor. MEFs derived from PKR-null mice remain insensitive to killing by this antibody when

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treated with dsRNA (S Der & BRG Williams, unpublished data). A role for Fas in virus-induced apoptosis has been suggested for influenza virus (294, 295), and it is likely that PKR is required, although experiments to prove this point remain to be carried out.

RNase L and 2-5A Synthetase

The possible wider role of the 2-5A system in cell metabolism extends beyond the antiviral activity of IFNs. The 2-5A system has been implicated in the action of IFNs. Although RNase L is not essential for normal mouse development (140), the 2-5A system has long been suspected to be involved in RNA decay during cell death. The regression of chick oviducts upon estrogen withdrawal and of rat mammary glands after lactation was correlated with the induction of 2-5A synthetase or with 2-5A per se (296-298). RNase L-null mice have enlarged thymus glands as a result of a defect in apoptosis (140). Thymocytes from these mice were resistant to inducers of apoptosis anti-CD3, anti-fas, staurosporine, and TNF α plus actinomycin D, whereas RNase L-null fibroblasts were resistant to staurosporine or the combination of IFN α and 2-5A. Expression of a dominant negative derivative of RNase L also suppressed apoptosis in cultured cells (J Castelli, BA Hassel, J Paranjape, A Maran, RH Silverman & R Youle, unpublished data). These findings suggest that the control of RNA stability by RNase L plays a role in apoptosis.

An intriguing but unresolved question is whether the 2-5A synthetases do something other than synthesize activators of RNase L. These enzymes differ in structure, intracellular location, activation profiles, and lengths of the 2-5A oligomers produced (127, 130, 299-302). The 2-5A synthetases are versatile enzymes that not only produce 2-5A but also transfer AMP residues in 2', 5'-linkage to a variety of molecules that terminate in an adenosine residue, such as A5'p35'A, A5'p45'A, NAD, ADP-ribose, and tRNA (303-307). Also, the final nucleotide added by 2-5A synthetase can be something other than AMP (302, 304). Recently, 2-5A synthetases have been used to make pppG2'p5'G by using GTP as a (relatively poor) substrate (302). 2', 5'-Oligoadenylates with structures different from 2-5A have been observed in cells and tissues of higher vertebrates (298), and some virus-induced alternative 2', 5'-oligoadenylates can function as inhibitors of RNase L (308). In summary, suggestions of a wider role for the 2-5A synthetases are tantalizing but remain largely unexplored.

IRFs

The IRF family of DNA-binding transcription factors, including IRF1, IRF2, IRF3, ISGF3 γ (p48), ICSBP, and ICSAT/PiP/LSIRF, has been implicated in IFN production, cell growth regulation, and induction of gene expression by IFN (100, 309, 310). Experiments using mice null for IRF family members

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have recently complemented studies of transfected cell lines and have also provided a link to PKR. IRF1 is essential for mouse *gfp* gene induction by IFN γ (311), and PKR is a signal transducer in this pathway (279). In the absence of PKR, IRF1 DNA binding activity induced in response to IFN γ (or LPS, TNF α , or dsRNA) is deficient. However, certain phenotypes and cellular responses of PKR- and IRF1-null mice are distinct, suggesting both shared and nonoverlapping pathways. For example, IRF1-null mice exhibit reduced levels of CD8 $^{+}$ T cells resulting from a failure of IFN γ to appropriately up-regulate the LMP1 and TAP2 genes, essential for class I MHC function (312–314). Recently it has been shown that IRF1 is required for a TH1 response in vivo (315,316). PKR-null mice have a normal complement of CD8 $^{+}$ cells in the periphery but exhibit exaggerated contact hypersensitivity, possibly because they fail to induce fas-dependent apoptosis appropriately (121, 187; S Kadereit, R Fairchild & BRG Williams, unpublished data). Both IRF1 and PKR appear to be essential for induction of the inducible nitric oxide synthase gene by IFN γ (317), but unlike IRF1, PKR is not involved in the induction of cell-cycle arrest in response to DNA damage (318; S Der, M Zamanian-Daryouch & BRG Williams, unpublished data). The phenotype of ICSBP-null mice (a lymphoid-specific member of the IRF1 family) is enhanced susceptibility to virus infection as a result of a deficiency in IFN γ production and a chronic myelogenous leukemia-like syndrome that is apparent even in the heterozygotes, suggesting haploin sufficiency (319). Because this phenotype is not shared with the IRF1 or PKR knockouts, ICSBP has a unique role in regulating hematopoiesis.

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ogy), respectively. Staining specificity was controlled by single staining, as well as by using secondary antibodies in the absence of the primary stain.

Generation of target cells

Target cells displaying a membrane-integral version of either wild-type HEL or a mutant¹⁰ exhibiting reduced affinity for HyHEL10 ([R²¹, D¹⁰¹, G¹⁰², N¹⁰³] designated HEL*) were generated by transfecting mouse J558L plasmacytoma cells with constructs analogous to those used¹⁰ for expression of soluble HEL/HEL*, except that 14 Ser/Gly codons, the H2K^b transmembrane region, and a 23-codon cytoplasmic domain were inserted immediately upstream of the termination codon by polymerase chain reaction. For mHEL-GFP, we included the EGFP coding domain in the Ser/Gly linker. Abundance of surface HEL was monitored by flow cytometry and radiolabelled-antibody binding using HyHEL5 and D1.3 HEL-specific monoclonal antibodies, for which the mutant HELs used in this work show unaltered affinities¹⁰.

Interaction assays

For B-cell/target interaction assays, splenic B cells from 3-83 or MD4 transgenic mice^{28,29} carrying (IgM + IgD) BCRs specific for HEL or H2K^b/H2K^b were freshly purified on Lympholyte and incubated with a twofold excess of target cells in RPMI, 50 mM HEPES pH 7.4, for the appropriate time at 37 °C before being applied to polylysine-coated slides. Cells were fixed in 4% paraformaldehyde/PBS or methanol and permeabilized with PBS/0.1% Triton X-100 before immunofluorescence. We acquired confocal images using a Nikon E800 microscope attached to BioRad Radiance Plus scanning system equipped with 488-nm and 543-nm lasers, as well as differential interference contrast for transmitted light. GFP fluorescence in living cells in real time was visualized using a Radiance 2000 and Nikon E300 inverted microscope. Images were processed using BioRad Lasersharp 1024 or 2000 software to provide single plane images, confocal projections or slicing.

Antigen presentation

Presentation of HEL epitopes to T-cell hybridomas 2G7 (specific for I-E^k[HEL¹⁻¹⁸]) and 1E5 (specific for I-E^d[HEL¹⁰⁸⁻¹¹⁶]) by transfectants of the LK35.2 B-cell hybridoma expressing an HEL-specific IgM BCR was monitored as described¹⁰.

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Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells

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RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene^{1–4}. The mediators of sequence-specific messenger RNA degradation are 21- and 22-nucleotide small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from longer dsRNAs^{5–9}. Here we show that 21-nucleotide siRNA duplexes specifically suppress expression of endogenous and heterologous genes in different mammalian cell lines, including human embryonic kidney (293) and HeLa cells. Therefore, 21-nucleotide siRNA duplexes provide a new tool for studying gene function in mammalian cells and may eventually be used as gene-specific therapeutics.

Uptake of dsRNA by insect cell lines has previously been shown to 'knock-down' the expression of specific proteins, owing to sequence-specific, dsRNA-mediated mRNA degradation^{6,10–12}. However, it has not been possible to detect potent and specific RNA interference in commonly used mammalian cell culture systems, including 293 (human embryonic kidney), NIH/3T3 (mouse fibroblast), BHK-21 (Syrian baby hamster kidney), and CHO-K1 (Chinese hamster ovary) cells, applying dsRNA that varies in size between 38 and 1,662 base pairs (bp)^{10,12}. This apparent lack of RNAi in mammalian cell culture was unexpected, because RNAi exists in mouse oocytes and early embryos^{13,14}, and because RNAi-related, transgene-mediated co-suppression was also observed in cultured Rat-1 fibroblasts¹⁵. But it is known that dsRNA in the cytoplasm of mammalian cells can trigger profound physiological

reactions that lead to the induction of interferon synthesis¹⁶. In the interferon response, dsRNA > 30 bp binds and activates the protein kinase PKR¹⁷ and 2',5'-oligoadenylate synthetase (2',5'-AS)¹⁸. Activated PKR stalls translation by phosphorylation of the translation initiation factors eIF2 α , and activated 2',5'-AS causes mRNA degradation by 2',5'-oligoadenylate-activated ribonuclease L. These responses are intrinsically sequence-nonspecific to the inducing dsRNA.

Base-paired 21- and 22-nucleotide (nt) siRNAs with overhanging 3' ends mediate efficient sequence-specific mRNA degradation in lysates prepared from *Drosophila* embryos⁹. To test whether siRNAs are also capable of mediating RNAi in cell culture, we synthesized 21-nt siRNA duplexes with symmetric 2-nt 3' overhangs directed against reporter genes coding for sea pansy (*Renilla reniformis*, RL) and two sequence variants of firefly (*Photinus pyralis*, GL2 and GL3) luciferases (Fig. 1a, b). The siRNA duplexes were co-transfected with the reporter plasmid combinations pGL2/pRL or pGL3/pRL, into *Drosophila* S2 cells or mammalian cells using cationic liposomes. Luciferase activities were determined 20 h after transfection. In *Drosophila* S2 cells (Fig. 2a and b), the specific inhibition of luciferases was complete and similar to results previously obtained for longer dsRNAs^{9,10,12,19}. In mammalian cells, where the reporter genes were 50- to 100-fold more strongly expressed, the specific suppression was less complete (Fig. 2c-j). In NIH/3T3, monkey COS-7 and HeLa S3 cells (Fig. 2c-h), GL2 expression was reduced 3-

to 12-fold, GL3 expression 9- to 25-fold, and RL expression 2- to 3-fold, in response to the cognate siRNAs. For 293 cells, targeting of RL luciferase by RL siRNAs was ineffective, although GL2 and GL3 targets responded specifically (Fig. 2i and j). The lack of reduction of RL expression in 293 cells may be because of its expression, 5- to 20-fold higher than any other mammalian cell line tested and/or to limited accessibility of the target sequence due to RNA secondary structure or associated proteins. Nevertheless, specific targeting of GL2 and GL3 luciferase by the cognate siRNA duplexes indicated that RNAi is also functioning in 293 cells.

The 2-nucleotide 3' overhang in all siRNA duplexes was composed of (2'-deoxy) thymidine, except for uGL2, which contained

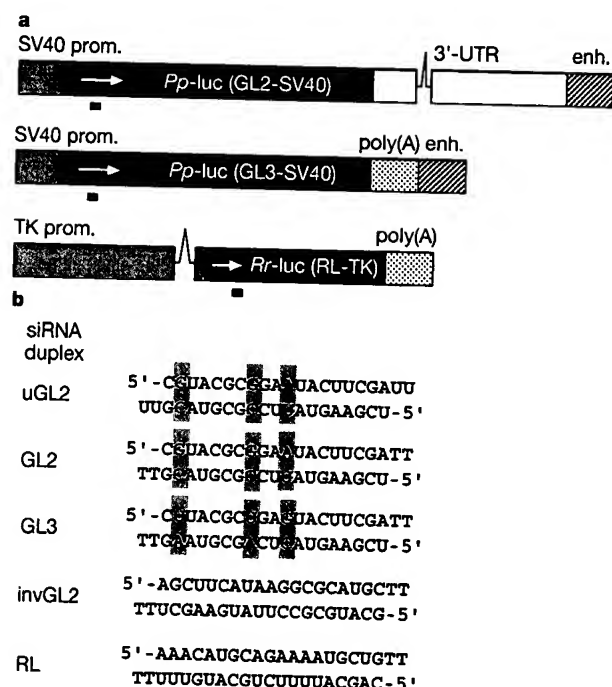


Figure 1 Reporter constructs and siRNA duplexes. **a**, The firefly (*Pp-luc*) and sea pansy (*Rr-luc*) luciferase reporter-gene regions from plasmids pGL2-Control, pGL3-Control, and pRL-TK (Promega) are illustrated; simian virus 40 (SV40) promoter (prom.); SV40 enhancer element (enh.); SV40 late polyadenylation signal (poly(A)); herpes simplex virus (HSV) thymidine kinase promoter, and two introns (lines) are indicated. The sequence of GL3 luciferase is 95% identical to GL2, but RL is completely unrelated to both. Luciferase expression from pGL2 is approximately 10-fold lower than from pGL3 in transfected mammalian cells. The region targeted by the siRNA duplexes is indicated as black bar below the coding region of the luciferase genes. **b**, The sense (top) and antisense (bottom) sequences of the siRNA duplexes targeting GL2, GL3, and RL luciferase are shown. The GL2 and GL3 siRNA duplexes differ by only three single-nucleotide substitutions (boxed in grey). As nonspecific control, a duplex with the inverted GL2 sequence, invGL2, was synthesized. The 2-nucleotide 3' overhang of 2'-deoxythymidine is indicated as TT; uGL2 is similar to GL2 siRNA but contains ribo-uridine 3' overhangs.

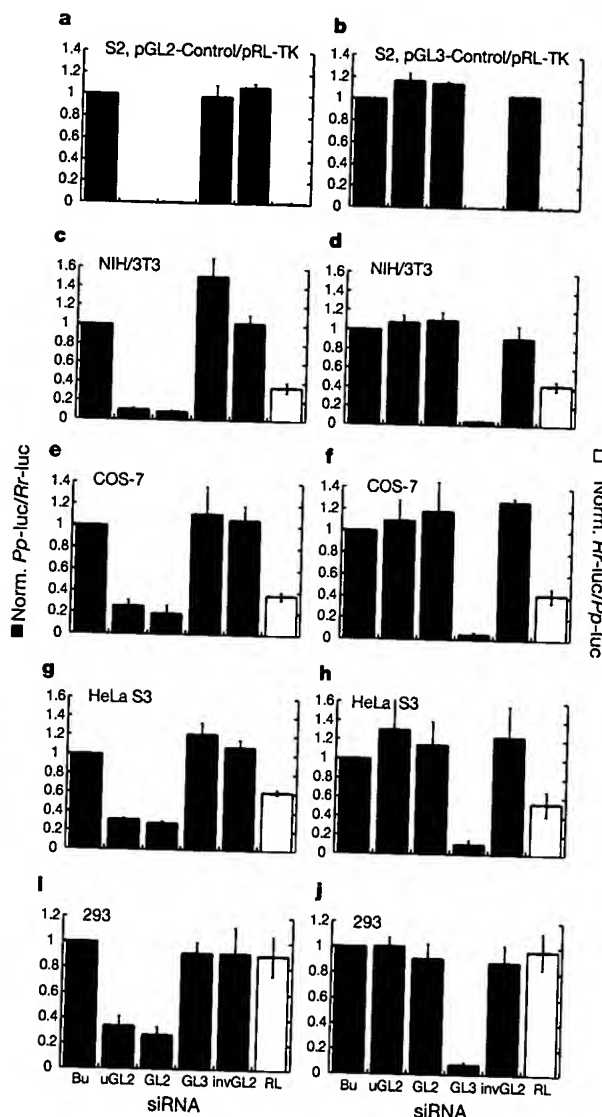


Figure 2 RNA interference by siRNA duplexes. Ratios of target to control luciferase were normalized to a buffer control (Bu, black bars); grey bars indicate ratios of *Photinus pyralis* (*Pp-luc*) GL2 or GL3 luciferase to *Renilla reniformis* (*Rr-luc*) RL luciferase (left axis), white bars indicate RL to GL2 or GL3 ratios (right axis). **a, c, e, g, i**, Experiments performed with the combination of pGL2-Control and pRL-TK reporter plasmids; **b, d, f, h, j**, experiments performed with the combination of pGL3-Control and pRL-TK reporter plasmids. The cell line used for the interference experiment is indicated at the top of each plot. The ratios of *Pp-luc*/*Rr-luc* for the buffer control (Bu) varied between 0.5 and 10 for pGL2/pRL, and between 0.03 and 1 for pGL3/pRL, respectively, before normalization and between the various cell lines tested. The plotted data were averaged from three independent experiments \pm s.d.

uridine residues. The thymidine overhang was chosen because it reduces costs of RNA synthesis and may enhance nuclease resistance of siRNAs in the cell culture medium and within transfected cells. As in the *Drosophila in vitro* system (data not shown), substitution of uridine by thymidine in the 3' overhang was well tolerated in cultured mammalian cells (Fig. 2a, c, e, g and i), and the sequence of the overhang appears not to contribute to target recognition⁹.

In co-transfection experiments, 25 nM siRNA duplexes were used (Figs 2 and 3; concentration is in respect to the final volume of tissue culture medium). Increasing the siRNA concentration to 100 nM did not enhance the specific silencing effects, but started to affect transfection efficiencies, perhaps due to competition for liposome encapsulation between plasmid DNA and siRNA (data not shown). Decreasing the siRNA concentration to 1.5 nM did not reduce the specific silencing effect (data not shown), even though the siRNAs were now only 2- to 20-fold more concentrated than the DNA plasmids; the silencing effect only vanishes completely if the siRNA concentration was dropped below 0.05 nM. This indicates that siRNAs are extraordinarily powerful reagents for mediating gene silencing, and that siRNAs are effective at concentrations that are several orders of magnitude below the concentrations applied in conventional antisense or ribozyme gene-targeting experiments²⁰.

To monitor the effect of longer dsRNAs on mammalian cells, 50- and 500-bp dsRNAs that are cognate to the reporter genes were prepared. As a control for nonspecific inhibition, dsRNAs from humanized GFP (hG)²¹ was used. In these experiments, the reporter plasmids were co-transfected with either 0.21 µg siRNA duplexes or 0.21 µg longer dsRNAs. The siRNA duplexes only reduced the expression of their cognate reporter gene, while the longer dsRNAs strongly and nonspecifically reduced reporter-gene expression. The effects are illustrated for HeLa S3 cells as a representative example (Fig. 3a and b). The absolute luciferase activities were decreased nonspecifically 10- to 20-fold by 50-bp dsRNA, and 20- to 200-fold by 500-bp dsRNA co-transfection, respectively. Similar nonspecific effects were observed for COS-7 and NIH/3T3 cells. For 293 cells, a 10- to 20-fold nonspecific reduction was observed only for 500-bp dsRNAs. Nonspecific reduction in reporter-gene expression by dsRNA > 30 bp was expected as part of the interferon response¹⁶. Interestingly, superimposed on the nonspecific interferon response, we detect additional sequence-specific, dsRNA-mediated silencing. The sequence-specific silencing effect of long dsRNAs, however, became apparent only when the relative reporter-gene activities were normalized to the hG dsRNA controls (Fig. 3c). Sequence-specific silencing by 50- or 500-bp dsRNAs reduced the targeted reporter-gene expression by an additional 2- to 5-fold. Similar effects were also detected in the other three mammalian cell lines tested (data not shown). Specific silencing effects with dsRNAs (356–1,662 bp) were previously reported in CHO-K1 cells, but the amounts of dsRNA required to detect a 2- to 4-fold specific reduction were about 20-fold higher than in our experiments¹². Also, CHO-K1 cells appear to be deficient in the interferon response. In another report, 293, NIH/3T3 and BHK-21 cells were tested for RNAi using luciferase/β-galactosidase (lacZ) reporter combinations and 829-bp specific lacZ or 717-bp nonspecific green fluorescent protein (GFP) dsRNA¹⁰. The lack of detected RNAi in this case may be due to the less sensitive luciferase/lacZ reporter assay and the length differences of target and control dsRNA. Taken together, our results indicate that RNAi is active in mammalian cells, but that the silencing effect is difficult to detect if the interferon system is activated by dsRNA > 30 bp.

To test for silencing of endogenous genes, we chose four genes coding for cytoskeletal proteins: lamin A/C, lamin B1, nuclear mitotic apparatus protein (NuMA) and vimentin²⁷. The selection was based on the availability of antibodies needed to quantitate the silencing effect. Silencing was monitored 40 to 45 h after transfection to allow for turnover of the protein of the targeted genes. As

shown in Fig. 4, the expression of lamin A/C was specifically reduced by the cognate siRNA duplex (Fig. 4a), but not when nonspecific siRNA directed against firefly luciferase (Fig. 4b) or buffer (Fig. 4c) was used. The expression of a non-targeted gene, NuMA, was unaffected in all treated cells (Fig. 4d–f), demonstrating the integrity of the targeted cells. The reduction in lamin A/C proteins was more than 90% complete as quantified by western blotting (Fig. 4j, k). We note that lamin A/C 'knock-out' mice are

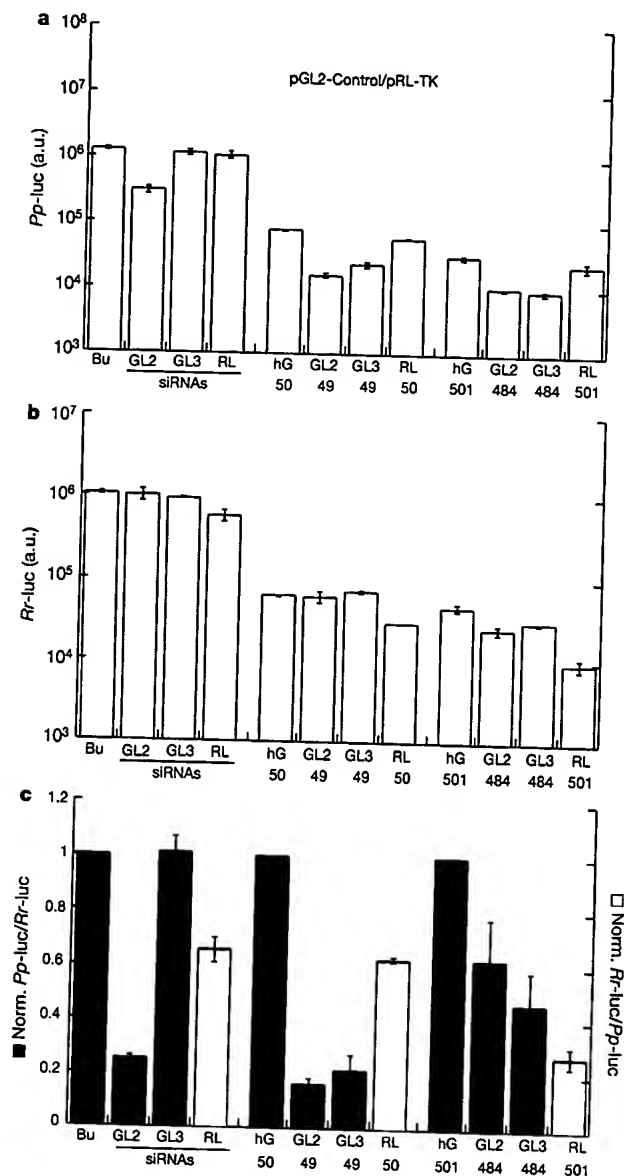


Figure 3 Effects of 21-nucleotide siRNAs, 50-bp, and 500-bp dsRNAs on luciferase expression in HeLa cells. The exact length of the long dsRNAs in base pairs is indicated below the bars. Experiments were performed with pGL2-Control and pRL-TK reporter plasmids. The data were averaged from two independent experiments \pm s.d. **a**, Absolute Pp-luc expression, plotted in arbitrary luminescence units (a.u.). **b**, Rr-luc expression, plotted in arbitrary luminescence units. **c**, Ratios of normalized target to control luciferase. The ratios of luciferase activity for siRNA duplexes were normalized to a buffer control (Bu, black bars); the luminescence ratios for 50- or 500-bp dsRNAs were normalized to the respective ratios observed for 50- and 500-bp dsRNAs from humanized GFP (hG, black bars). We note that the overall differences in sequence between the 49- and 484-bp GL2 and GL3 dsRNAs are not sufficient to confer specificity for targeting GL2 and GL3 targets (43-nucleotide uninterrupted identity in 49-bp segment, 239-nucleotide longest uninterrupted identity in 484-bp segment)³⁰.

viable for a few weeks after birth²³ and that the lamin A/C knock-down in cultured cells was not expected to cause cell death. Lamin A and C are produced by alternative splicing in the 3' region and are present in equal amounts in the lamina of mammalian cells (Fig. 4j, k). Transfection of siRNA duplexes targeting lamin B1 and NuMA reduced the expression of these proteins to low levels (data not shown), but we were not able to observe a reduction in vimentin expression. This could be due to the high abundance of vimentin in the cells (several per cent of total cell mass) or because the siRNA sequence chosen was not optimal for targeting of vimentin.

The mechanism of the 21-nucleotide siRNA-mediated interference process in mammalian cells remains to be uncovered, and silencing might occur post-transcriptionally and/or transcriptionally. In *Drosophila* lysate, siRNA duplexes mediate post-transcriptional gene silencing by reconstitution of siRNA-protein complexes (siRNPs), which guide mRNA recognition and targeted cleavage^{6,7,9}. In plants, dsRNA-mediated post-transcriptional silencing has also been linked to DNA methylation, which may also be directed by 21-

nucleotide siRNAs²⁴. Methylation of promoter regions can lead to transcriptional silencing²⁵, but methylation in coding sequences does not²⁶. DNA methylation and transcriptional silencing in mammals are well documented processes²⁷, yet their mechanisms have not been linked to that of post-transcriptional silencing. Methylation in mammals is predominantly directed towards CpG dinucleotide sequences. There is no CpG sequence in the RL or lamin A/C siRNA, although both siRNAs mediate specific silencing in mammalian cell culture, so it is unlikely that DNA methylation is essential for the silencing process.

Thus we have shown, for the first time, siRNA-mediated gene silencing in mammalian cells. The use of exogenous 21-nucleotide siRNAs holds great promise for analysis of gene function in human cell culture and the development of gene-specific therapeutics. It will also be of interest in understanding the potential role of endogenous siRNAs in the regulation of mammalian gene function. □

Methods

RNA preparation

21-nucleotide RNAs were chemically synthesized using Expedite RNA phosphoramidites and thymidine phosphoramidite (Proligo, Germany). Synthetic oligonucleotides were deprotected and gel-purified⁹. The accession numbers given below are from GenBank. The siRNA sequences targeting GL2 (Acc. No. X65324) and GL3 luciferase (Acc. No. U47296) corresponded to the coding regions 153–173 relative to the first nucleotide of the start codon; siRNAs targeting RL (Acc. No. AF025846) corresponded to region 119–139 after the start codon. The siRNA sequence targeting lamin A/C (Acc. No. X03444) was from position 608–630 relative to the start codon; lamin B1 (Acc. No. NM_005573) siRNA was from position 672–694; NuMA (Acc. No. Z11583) siRNA from position 3,988–4,010, and vimentin (Acc. No. NM_003380) from position 346–368 relative to the start codon. Longer RNAs were transcribed with T7 RNA polymerase from polymerase chain reaction (PCR) products, followed by gel purification. The 49- and 484-bp GL2 or GL3 dsRNAs corresponded to positions 113–161 and 113–596, respectively, relative to the start of translation; the 50- and 501-bp RL dsRNAs corresponded to position 118–167 and 118–618, respectively. PCR templates for dsRNA synthesis targeting humanized GFP (hG) were amplified from pAD3 (ref. 21), whereby 50- and 501-bp hG dsRNA corresponded to positions 121–170 and 121–621, respectively, to the start codon.

For annealing of siRNAs, 20 μ M single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90 °C followed by 1 h at 37 °C. The 37 °C incubation step was extended overnight for the 50- and 500-bp dsRNAs, and these annealing reactions were performed at 8.4 μ M and 0.84 μ M strand concentrations, respectively.

Cell culture

S2 cells were propagated in Schneider's *Drosophila* medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) 100 units ml⁻¹ penicillin, and 100 μ g ml⁻¹ streptomycin at 25 °C. 293, NIH/3T3, HeLa S3, HeLa S6, COS-7 cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units ml⁻¹ penicillin, and 100 μ g ml⁻¹ streptomycin. Cells were regularly passaged to maintain exponential growth. Twenty-four h before transfection at 50–80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics (1–3 \times 10⁵ cells ml⁻¹) and transferred to 24-well plates (500 μ l per well). S2 cells were not trypsinized before splitting. Co-transfection of reporter plasmids and siRNAs was carried out with Lipofectamine 2000 (Life Technologies) as described by the manufacturer for adherent cell lines. Per well, 1.0 μ g pGL2-Control (Promega) or pGL3-Control (Promega), 0.1 μ g pRL-TK (Promega), and 0.21 μ g siRNA duplex or dsRNA, formulated into liposomes, were applied; the final volume was 600 μ l per well. Cells were incubated 20 h after transfection and appeared healthy thereafter. Luciferase expression was subsequently monitored with the Dual luciferase assay (Promega). Transfection efficiencies were determined by fluorescence microscopy for mammalian cells lines after co-transfection of 1.1 μ g hGFP-encoding pAD3 (ref. 21) and 0.21 μ g inverted GL2 siRNA, and were 70–90%. Reporter plasmids were amplified in XL-1 Blue (Stratagene) and purified using the Qiagen EndoFree Maxi Plasmid Kit.

Transfection of siRNAs for targeting endogenous genes was carried out using Lipofectamine (Life Technologies) and 0.84 μ g siRNA duplex per well, but it was recently found that as little as 0.01 μ g siRNAs per well are sufficient to mediate silencing. HeLa S6 cells were transfected one to three times in approximately 15 h intervals and were assayed 40 to 45 h after the first transfection. It appears, however, that a single transfection is as efficient as multiple transfections. Transfection efficiencies as determined by immunofluorescence of targeted cells were in the range of 90%. Specific silencing of targeted genes was confirmed by at least three independent experiments.

Western blotting and immunofluorescence microscopy

Monoclonal 636 lamin A/C specific antibody²⁸ was used as undiluted hybridoma supernatant for immunofluorescence and 1/100 dilution for western blotting. Affinity-purified polyclonal NuMA protein 705 antibody²⁹ was used at a concentration of 10 μ g ml⁻¹ for

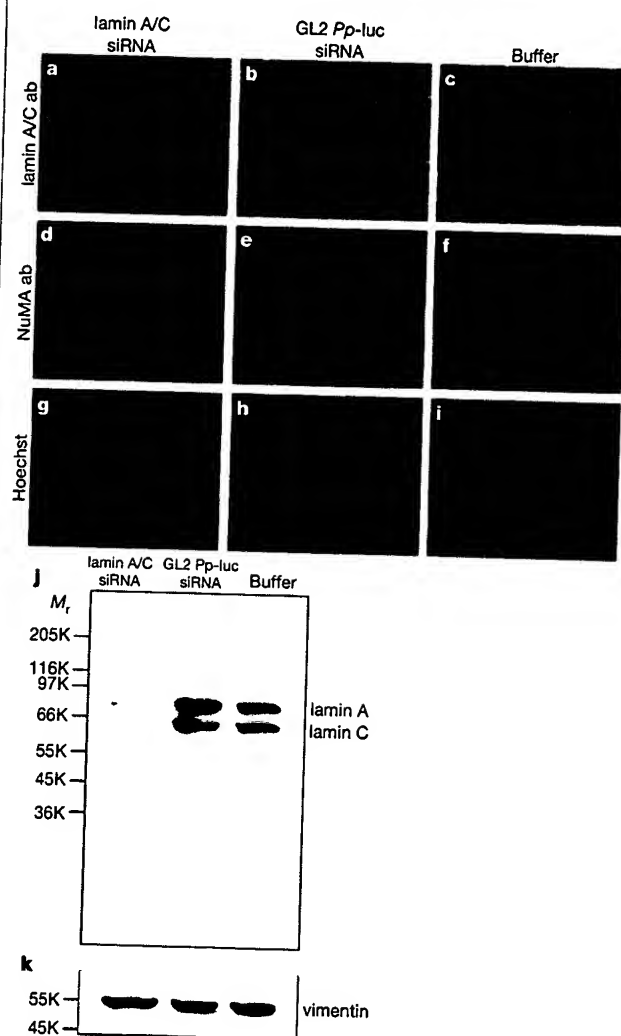


Figure 4 Silencing of nuclear envelope proteins lamin A/C in HeLa cells. Triple fluorescence staining of cells transfected with lamin A/C siRNA duplex (a, d, g), with GL2 luciferase siRNA duplex (nonspecific siRNA control) (b, e, h), and with buffer only (c, f, i). a–c, Staining with lamin A/C specific antibody; d–f, staining with NuMA-specific antibody; g–i, Hoechst staining of nuclear chromatin. Bright fluorescent nuclei in a represent untransfected cells. j, k, Western blots of transfected cells using lamin A/C- (j) or vimentin-specific (k) antibodies. The Western blot was stripped and re-probed with vimentin antibody to check for equal loading of total protein.

immunofluorescence. Monoclonal V9 vimentin-specific antibody was used at 1/2,000 dilution. For western blotting, transfected cells grown in 24-well plates were trypsinized and harvested in SDS sample buffer. Equal amounts of total protein were separated on 12.5% polyacrylamide gels and transferred to nitrocellulose. Standard immunostaining was carried out using ECL enhanced chemiluminescence technique (Amersham Pharmacia).

For immunofluorescence, transfected cells grown on glass coverslips in 24-well plates were fixed in methanol for 6 min at -10°C . Target gene specific and control primary antibody were added and incubated for 80 min at 37°C . After washing in phosphate buffered saline (PBS), Alexa 488-conjugated anti-rabbit (Molecular Probes) and Cy3-conjugated anti-mouse (Dianova) antibodies were added and incubated for 60 min at 37°C . Finally, cells were stained for 4 min at room temperature with Hoechst 33342 ($1\ \mu\text{M}$ in PBS) and embedded in Mowiol 488 (Hoechst). Pictures were taken using a Zeiss Axiophot camera with a Fluor 40/1.30 oil objective and MetaMorph Imaging Software (Universal Imaging Corporation) with equal exposure times for the specific antibodies.

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Ribosomal peptidyl transferase can withstand mutations at the putative catalytic nucleotide

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Peptide bond formation is the principal reaction of protein synthesis. It takes place in the peptidyl transferase centre of the large (50S) ribosomal subunit. In the course of the reaction, the polypeptide is transferred from peptidyl transfer RNA to the α -amino group of amino acyl-tRNA. The crystallographic structure of the 50S subunit showed no proteins within 18 Å from the active site, revealing peptidyl transferase as an RNA enzyme¹. Reported unique structural and biochemical features of the universally conserved adenine residue A2451 in 23S ribosomal RNA (*Escherichia coli* numbering) led to the proposal of a mechanism of rRNA catalysis that implicates this nucleotide as the principal catalytic residue^{2,3}. *In vitro* genetics allowed us to test the importance of A2451 for the overall rate of peptide bond formation. Here we report that large ribosomal subunits with mutated A2451 showed significant peptidyl transferase activity in several independent assays. Mutations at another nucleotide, G2447, which is essential to render catalytic properties to A2451 (refs 2, 3), also did not dramatically change the transpeptidation activity. As alterations of the putative catalytic residues do not severely affect the rate of peptidyl transfer the ribosome apparently promotes transpeptidation not through chemical catalysis, but by properly positioning the substrates of protein synthesis. The proposed role of A2451 in the peptidyl transfer reaction is

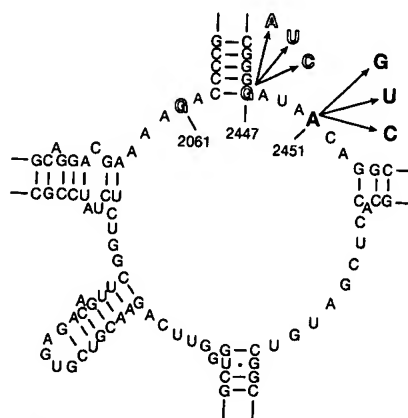


Figure 1 The secondary structure of the central loop of domain V of *T. aquaticus* 23S rRNA. Position A2451 (*E. coli* 23S rRNA numbering), the principal catalytic nucleotide in the proposed general acid–base catalytic mechanism of peptide bond formation^{2,3}, is shown in bold. Its tertiary interaction partners, guanine residues 2061 and 2447, suggested to be essential for rendering catalytic properties to A2451, are outlined. Arrows indicate the mutations engineered in 23S rRNA.

Involvement of the double-stranded-RNA-dependent kinase PKR in interferon expression and interferon-mediated antiviral activity

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ABSTRACT The signaling mechanisms responsible for the induced expression of interferon (IFN) genes by viral infection or double-stranded RNA (dsRNA) are not well understood. Here we investigate the role of the interferon-induced dsRNA-dependent protein kinase PKR in the regulation of IFN induction. Biological activities attributed to PKR include regulating protein synthesis, mediating IFN actions, and functioning as a possible tumor suppressor. Since binding of dsRNA is required for its activation, PKR has been considered as a candidate signal transducer for regulating IFN expression. To examine this role of PKR, loss-of-function phenotypes in stable transformants of promonocytic U-937 cells were achieved by two different strategies, overexpression of an antisense PKR transcript or a dominant negative PKR mutant gene. Both types of PKR-deficient cells were more permissive for viral replication than the control U-937 cells. As the result of PKR loss, they also showed impaired induction of IFN- α and IFN- β genes in response to several inducers—specifically, encephalomyocarditis virus, lipopolysaccharide, and phorbol 12-myristate 13-acetate. Interestingly, while IFN- α induction by dsRNA was impaired in PKR-deficient cells, IFN- β induction remained intact. Loss of PKR function also resulted in decreased antiviral activity as elicited by IFN- α and, to a greater extent, by IFN- γ . These results implicate PKR in the regulation of several antiviral activities.

Type I interferons (IFNs) regulate diverse biological processes including antiviral activities, cellular growth and differentiation, and modulation of immune functions (1, 2). The induced expression of type I IFN genes, which include the IFN- α and IFN- β gene families, is detected typically following viral infections. Previous studies have identified promoter elements and transcription factors involved in regulating the expression of type I IFNs (3–5). However, it remains unclear what are the particular biochemical cues that signify viral infections to the cell and the signaling mechanisms involved. Since many forms of double-stranded RNA (dsRNA) are capable of inducing type I IFNs, this led to suggestions that the common inducing molecule among different viruses was a viral replicative intermediate containing dsRNA (6). It seems reasonable, therefore, to hypothesize that the regulation of IFN genes and antiviral activities involves effector proteins responsive to dsRNA.

Of the many RNA-binding proteins, the few which are capable of binding dsRNA are distinguished by a conserved 65- to 68-amino acid “dsRNA-binding domain” (7). Among these, the IFN-induced dsRNA-dependent protein kinase PKR is the only one with kinase function. PKR is a serine/threonine kinase whose enzymatic activation requires dsRNA binding and consequent autophosphorylation (8, 9). The best characterized substrate of PKR is the α subunit of eukaryotic initiation factor 2, which once phosphorylated leads to inhibition of cellular and viral protein synthesis (10). This function

of PKR has been suggested as one of the mechanisms responsible for mediating the antiviral and antiproliferative activities of IFNs. An additional putative function for PKR is its role as a signal transducer, since 2-aminopurine, a relatively specific inhibitor of PKR, can block the induction of IFN- α and IFN- β genes by viral infection or dsRNA (11, 12). In support of this, Kumar *et al.* (13) have demonstrated that PKR can phosphorylate I κ B α , resulting in the release and activation of the transcription factor NF- κ B (13). Given the well-characterized NF- κ B site in the IFN- β promoter and that dsRNA alone can induce NF- κ B activity (14), it has been postulated that PKR mediates the induction of IFN- β transcription by dsRNA.

To investigate the role of PKR in IFN gene regulation and cellular antiviral responses, we have utilized two different strategies to achieve a loss-of-PKR-function phenotype. This involved overexpression of a dominant negative PKR mutant gene, encoding [Arg²⁹⁶]PKR, or an antisense PKR gene in stable transformants of a promonocytic cell line, U-937. Monocytes represent a primary source of type I IFNs *in vivo* and accordingly, we and others have found U-937 cells useful for studying IFN- α and IFN- β gene expression (15). The mutant [Arg²⁹⁶]PKR contains a single amino acid substitution of arginine for the invariant lysine in catalytic domain II at position 296 and is a dominant negative protein which can specifically suppress the activity of endogenous wild-type PKR *in vivo* (14, 15). An alternative approach to specifically inhibit gene expression involves antisense strategies. Recently, Maran *et al.* (16) showed that 2'-5'-oligoadenylate-linked antisense oligonucleotides specific for PKR suppressed PKR activity and NF- κ B activation by dsRNA. However, it is not known whether IFN production or IFN-mediated antiviral responses were affected as a result of suppressed PKR function in the above studies. Here we report that loss of PKR activity in U-937 cells results in multiple defects both in IFN production and in antiviral responsiveness to IFN- α and IFN- γ .

METHODS AND MATERIALS

Plasmids and Stable Transformants. The wild-type human PKR gene and the dominant negative [Arg²⁹⁶]PKR mutant gene were released by *Hind*III digestion from the plasmids pBSKS and p6M (provided by B. R. G. Williams, Cleveland Clinic Research Institute, Cleveland), respectively. They were then subcloned into the eukaryotic expression vector pRC-CMV (Invitrogen) to generate the plasmids used in this study, namely, pPKR-AS (antisense) and p[Arg²⁹⁶]PKR. Stable transformants were generated by electroporation of U-937 cells with 10 μ g of each plasmid by use of a Gene Pulser apparatus (Bio-Rad). Clonal lines were obtained by selection with Geneticin (400 μ g/ml; GIBCO/BRL) and limiting dilu-

Abbreviations: IFN, interferon; dsRNA, double-stranded RNA; EMCV, encephalomyocarditis virus; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; TCID₅₀, median tissue culture infective dose. †To whom reprint requests should be addressed.

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tion cloning. Cells were cultured in RPMI 1640 containing 10% fetal bovine serum and Geneticin.

PKR Analysis. The PKR autophosphorylation assay was performed essentially as described by Maran *et al.* (16), with the following modifications. Cell extracts (100 μ g) were incubated with poly(I)-poly(C)-cellulose for 1 hr on ice, washed three times, and incubated for 30 min at 30°C in 50 μ l of reaction buffer [20 mM Hepes, pH 7.5/50 mM KCl/5 mM 2-mercaptoethanol/1.5 mM Mg(OAc)₂/1.5 mM MnCl₂] containing 1 μ Ci (37 kBq) of [γ -³²P]ATP. Samples were analyzed by SDS/10% PAGE and autoradiography. For immunoblot analysis of PKR, cell extract proteins (100 μ g) were separated by SDS/10% PAGE and electrotransferred onto nitrocellulose membranes. Membranes were incubated with anti-PKR monoclonal antibody at 1:1000 in "Blotto" (5% nonfat dry milk/0.05% Tween 20 in Tris-buffered saline), with final detection provided by horseradish peroxidase-conjugated goat anti-mouse antibody (Santa Cruz Biotech) and a chemiluminescence method (ECL; Amersham).

Encephalomyocarditis Virus (EMCV) Replication and IFN Assay. For determination of EMCV replication, U-937-derived transformants were cultured in complete medium alone or pretreated with recombinant human IFN- α 2 (Schering) or IFN- γ (Amgen Biologicals) for 18 hr. Following two washes with phosphate-buffered saline, the cells were incubated with the indicated amounts of EMCV in serum-free medium for 2 hr. The cells were washed again and 10⁶ cells per sample were suspended in 1 ml of medium containing 1% fetal bovine serum. Samples were collected at the required time points and lysed by three rounds of freeze-thaw. Fourfold serial dilutions of the samples were added onto L929 mouse fibroblast monolayers and incubated for 48 hr before the monolayers were stained with 0.05% crystal violet to determine cytopathic effects and median tissue culture infective dose (TCID₅₀). In assays of IFN production, U-937-derived transformants were similarly pretreated with IFNs as described above. Then, the cells were incubated with inducers [poly(I)-poly(C) (Pharmacia), EMCV, lipopolysaccharide (LPS; Sigma), or phorbol 12-myristate 13-acetate (PMA; Sigma)] for 2 hr. Cells were washed and cultured in medium containing 1% fetal bovine serum. Supernatants were collected after 24 hr and IFN activity was measured by a bioassay (17).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Priming and induction of U-937-derived transformants were performed as above. Total RNA was extracted from cell samples by an acid guanidinium thiocyanate procedure. First-strand cDNA synthesis was performed with 2 μ g of each RNA sample primed with random hexamer in a 25- μ l reaction volume with 200 units of Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL). All PCRs were performed with 2 μ l out of each cDNA mixture in a 50- μ l reaction volume containing 50 pmol of each upstream and downstream primer, 2 units of *Taq* DNA polymerase (Promega), 0.2 mM each dNTP, 2.5 mM MgCl₂, and 10 \times reaction buffer. IFN- α PCR used consensus primers, capable of amplifying all 14 known human IFN- α subtype genes, 5'-GGAAGCTTCTCTGYYTGAWGGACAGA-3' and 5'-GGGGATCCTCTGCAACCTCCCANGCACA-3', which generate an expected product of 372 bp. IFN- β PCR used primers, 5'-GTGTCAG-AAGCTCCTGTGGC-3' and 5'-CTTCAGTTTCGGAGGT-AACC-3', which generate an expected product of 456 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR used primers, 5'-CCATGGAGAAGGCTGGGG-3' and 5'-CA-AAGTTGTGATGGATGACC-3', which generate an expected product of 196 bp.

RESULTS

Characterization of PKR-Deficient Stable Transformants. Stable transformant cell lines were obtained by transfecting

U-937 cells with the following expression plasmids. Five representative cell lines were selected for characterization: (i) U937-neo was the control cell line transfected with the parental vector, pRC/CMV; (ii) U937-AS1 and U937-AS3 were independent clones transfected with pPKR-AS; and (iii) U937-M13 and U937-M22 were independent clones transfected with p[Arg²⁹⁶]PKR. PKR kinase activity was measured with an assay that uses poly(I)-poly(C)-cellulose for binding and activation of PKR enzyme. IFN-treated HeLa and mouse L929 cells were used as positive controls (Fig. 1A, lanes 1 and 8), since PKR activity in these cells had been described (9). Similar to the untransfected U-937 parents, U937-neo cells contained basal levels of PKR activity which increased following treatment with IFN- α (Fig. 1A, lanes 2 and 3). In contrast, PKR activity was not detected in any of the four cell lines transformed with pPKR-AS or p[Arg²⁹⁶]PKR (results not shown). Furthermore, PKR activity was not restored in these cells by treatment with IFN- α (Fig. 1A, lanes 4–7) or IFN- γ (results not shown). To further confirm the inhibition of PKR expression in the pPKR-AS-transformed cells, Western blot analysis was performed with a monoclonal antibody specific for human PKR. Basal levels of PKR protein were detectable in U937-neo cells (Fig. 1B, lane 1), which increased following treatment with IFN- α or IFN- γ (lanes 2 and 3). In contrast, PKR expression was diminished in U937-AS1 and U937-AS3 cells (Fig. 1B, lanes 4 and 6) and did not increase with IFN- α treatment (lanes 5 and 7).

Enhanced EMCV Replication in PKR-Deficient Cells. We investigated whether loss of PKR function would affect the rate of EMCV replication. In control U937-neo cells following challenge with EMCV at 0.1 TCID₅₀ per cell, viral titers peaked at $\sim 10^4$ TCID₅₀/ml after 48 hr (Fig. 2A). However, in U937-AS1 and U937-M22 cells, EMCV replication was substantially higher, reaching titers of 10⁴–10⁵ TCID₅₀/ml after only 24 hr and 10⁸ TCID₅₀/ml by 48 hr, a 1000-fold increase over U937-neo cells. With a lower virus inoculum, 0.001

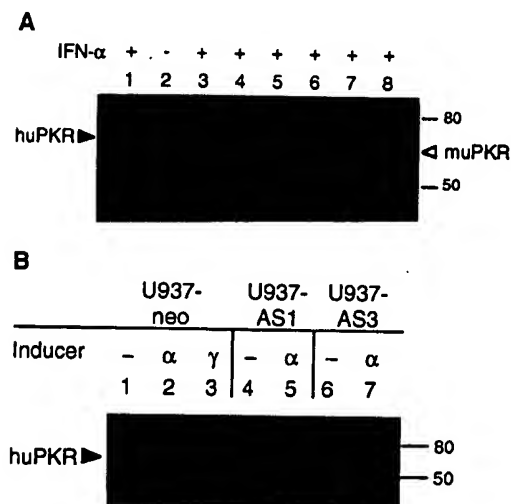


FIG. 1. PKR activity and protein levels in U-937-derived stable transformant cell lines. (A) PKR activity was determined by a poly(I)-poly(C)-cellulose assay for PKR autophosphorylation. Cell extracts were prepared from HeLa cells and the various U-937-derived cell lines following incubation with or without recombinant human IFN- α 2 (200 units/ml) as indicated, while L929 cells were similarly treated with mouse IFN- α / β . Lane 1, HeLa; lanes 2 and 3, U937-neo; lane 4, U937-AS1; lane 5, U937-AS3; lane 6, U937-M13; lane 7, U937-M22; lane 8, L929. Positions of the human (68 kDa) and murine (65 kDa) PKR proteins and the molecular size standards (80 and 50 kDa) are indicated. (B) Cell extracts were prepared as above after induction with IFN- α or - γ , and PKR protein levels were determined by Western blot analysis.

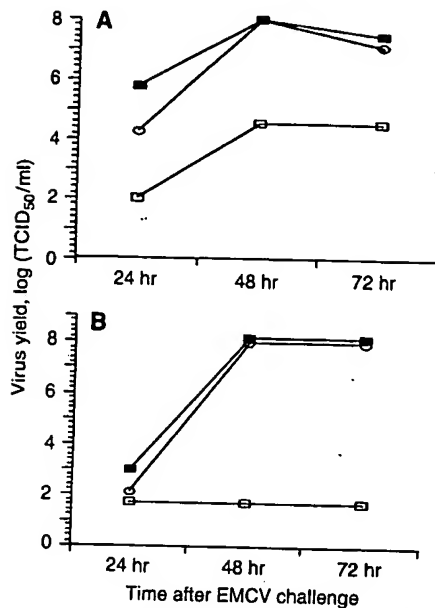


FIG. 2. Kinetics of EMCV replication are enhanced in PKR-deficient cells. The U937-neo (□), U937-M22 (○), and U937-AS1 (■) cell lines were challenged with EMCV at 0.1 (A) or 0.001 (B) TCID₅₀ per cell. Samples were harvested at the indicated times and viral yields were measured in terms of TCID₅₀.

TCID₅₀ per cell, more dramatic differences in EMCV susceptibility were observed. While EMCV replication in U937-neo cells did not exceed 10² TCID₅₀/ml, high viral titers of 10⁸

TCID₅₀/ml were attained in both U937-AS1 and U937-M22 cells (Fig. 2B).

A Role for PKR in IFN Expression. The commonly invoked model for IFN action proposes that an essential function for the IFNs secreted from virus-infected cells is to protect neighboring cells against subsequent rounds of infection by progeny virus (1, 2, 5). Accordingly, the higher rates of EMCV replication in the PKR-deficient cells could have resulted from impaired IFN production or defective antiviral responses to the paracrine actions of induced IFNs. Here, we first showed that loss of PKR activity resulted in impaired IFN production in both U937-AS1 and U937-M22 cells compared with the control U937-neo cells. With U937-neo cells, induction by EMCV alone produced substantial amounts of secreted IFN protein (512 units/ml; Fig. 3A). In a phenomenon known as IFN priming, pretreatment of the IFN-producer cells with even small amounts of IFN enhances subsequent IFN production upon stimulation with inducers (18). Consistent with this, priming of U937-neo cells with either IFN-α or IFN-γ resulted in increased production of EMCV-induced IFN activity (Fig. 3A). The effects of priming were more significant for IFN induction by nonviral inducers. Stimulation of U937-neo cells with poly(I):poly(C), LPS, or PMA alone did not induce any detectable levels of IFN unless the cells had been primed with IFN-α or IFN-γ (Fig. 3A). In contrast, IFN production was significantly impaired in both types of PKR-deficient cells under each of the above induction conditions (Fig. 3A). Compared with U937-neo cells, IFN levels from U937-AS1 and U937-M22 cells following EMCV induction were reduced nearly 50-fold (≤16 units/ml). Further, this impairment was not alleviated by IFN-α or IFN-γ priming. Also, IFN induction by poly(I):poly(C), LPS, or PMA, following IFN priming, was impaired as well in both PKR-deficient cell lines (≤8 units/ml). The IFN activity produced by U-937 cells was composed

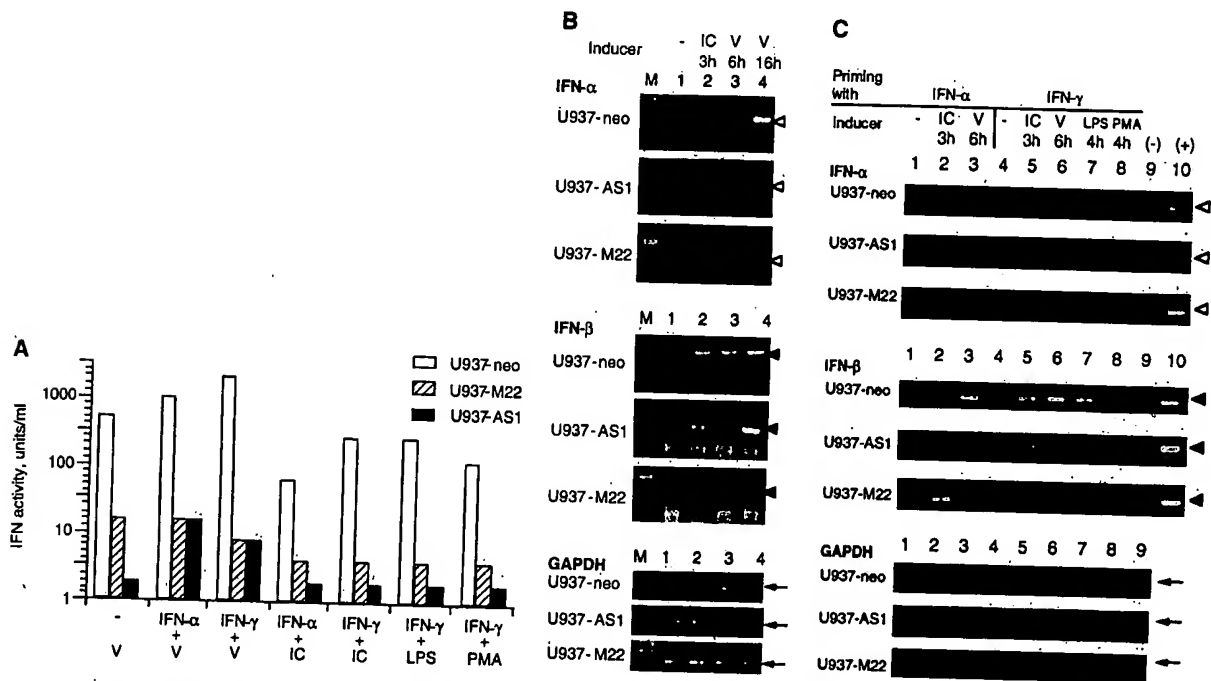


FIG. 3. IFN expression is impaired in PKR-deficient cells. (A) The different U937 cell lines were primed where specified with IFN-α or IFN-γ (200 units/ml). Cells were then incubated with the indicated inducers and IFN levels from each sample were determined. Following our induction conditions, U937 cells did not produce detectable IFN in response to poly(I):poly(C) (IC, 100 μg/ml), LPS (50 ng/ml), or PMA (50 nM) alone; or after IFN priming in the absence of inducers. V, EMCV. (B and C) Cells were primed as described above and then incubated with inducers for the indicated times. IFN-α, IFN-β, and GAPDH mRNA were detected by RT-PCR. PCR products were visualized by ethidium bromide staining after 1.5% agarose gel electrophoresis. Negative controls (-) represent PCR performed on RT reagents without sample RNA. Positive controls (+) represent PCR amplification of 1.0 ng of human genomic DNA. DNA markers (M) represent a ladder of 100-bp increments.

of both IFN- α (>80%) and IFN- β proteins, as determined with neutralizing antibodies (results not shown).

To examine the role of PKR in regulating the differential expression of IFN- α and IFN- β genes, steady-state levels of the respective IFN mRNAs were determined by RT-PCR. Optimal induction of IFN- α mRNA in U937-neo cells by EMCV alone required stimulation for 16 hr (Fig. 3B, lane 4). Peak induction of IFN- β mRNA by poly(I):poly(C) or EMCV alone was more rapid, occurring at 3 or 6 hr, respectively (Fig. 3B, lanes 2 and 3). In contrast, the induction of IFN- α and IFN- β mRNA following viral infection was impaired in both PKR-deficient cell lines. IFN- α mRNA induction by EMCV at 16 hr was diminished in U937-AS1 and U937-M22 cells (Fig. 3B, lane 4). Also, the early EMCV induction of IFN- β mRNA at 6 hr was absent (Fig. 3B, lane 3). However, IFN- β mRNA remained inducible in response to poly(I):poly(C) (Fig. 3B, lane 2), and various levels were induced by EMCV only after 16 hr (lane 4) in the PKR-deficient cell lines.

Next, the effect of PKR loss on the induction of IFN mRNAs was examined in IFN-primed cells. We have determined that priming enhances IFN- α induction in U-937 cells. Stimulation of U937-neo cells with poly(I):poly(C) alone resulted in weak IFN- α mRNA induction after 16 hr (results not shown). However, following priming with either IFN- α or IFN- γ , poly(I):poly(C) stimulation resulted in a rapid induction of IFN- α mRNA, peaking after 3 hr (Fig. 3C, lanes 2 and 5, versus Fig. 3B, lane 2). Similarly, IFN priming also enabled a rapid induction of IFN- α mRNA in response to EMCV, peaking after 6 hr (Fig. 3C, lanes 3 and 6 versus Fig. 3B, lane 3). Further, the induction of IFN- α mRNA in U937-neo cells by LPS or PMA was dependent on priming with IFN- γ (Fig. 3C, lanes 7 and 8). Consistent with the patterns observed earlier for IFN protein production, IFN mRNA expression was impaired in both PKR-deficient cell lines despite priming. The early induction of IFN- α and IFN- β mRNA following EMCV challenge was absent in both U937-AS1 and U937-M22 cells, irrespective of priming with IFN- α or IFN- γ . The induction of both IFN- α and IFN- β mRNA by LPS or PMA was also impaired in the PKR-deficient cells. While the induction of IFN- α mRNA by poly(I):poly(C) was diminished in the U937-AS1 and U937-M22 cells, IFN- β mRNA induction by poly(I):poly(C) again appeared unaffected (Fig. 3C, lanes 2 and 5).

Impaired IFN Responsiveness in PKR-Deficient Cells. Finally, we investigated whether loss of PKR activity affected IFN-induced antiviral responses. To test this, EMCV replication was measured after treatment of cells with IFN- α or IFN- γ . Although generally not produced by macrophages, IFN- γ was studied for its effects on U-937 cells since it possesses direct antiviral properties and has a primary role in macrophage activation (19). While treatment with IFNs reduced EMCV titers in all cell lines, viral yields were consistently higher in the PKR-deficient cells compared with the control cells (Fig. 4A). EMCV titers were 10-fold higher in both PKR-deficient cell lines than in U937-neo cells after IFN- α treatment. Interestingly, IFN- γ -mediated antiviral activity was more severely impaired as a result of PKR loss, since EMCV titers from IFN- γ -primed U937-AS1 or U937-M22 cells were 10^2 - to 10^3 -fold higher than those from control cells. We considered the possibility that these experimental conditions involving a relatively low virus inoculum may have magnified the differences in IFN-mediated antiviral responses between these cell lines. However, similar results were observed when we applied more stringent conditions for comparing IFN responsiveness, by increasing the EMCV inoculum 100-fold and harvesting samples earlier, at 24 hr rather than 48 hr (Fig. 4B).

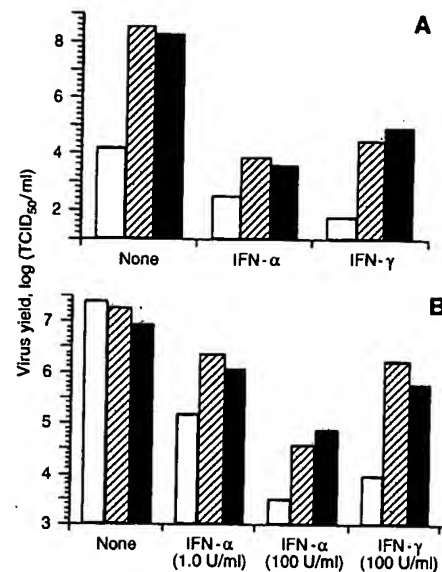


FIG. 4. Inhibition of EMCV replication by IFN- α or IFN- γ is impaired in PKR-deficient cells. (A) U937-neo (open bars), U937-AS1 (filled bars), and U937-M22 (hatched bars) cells were cultured in the absence or presence of the indicated concentrations of IFN- α or IFN- γ at 100 units/ml for 18 hr and challenged with EMCV at 0.1 TCID₅₀ per cell. Samples were harvested after 48 hr for determination of virus yield. (B) Cells (bars as in A) were similarly pretreated with or without IFNs [1 or 100 units (U)/ml], except they were challenged with EMCV at 10.0 TCID₅₀ per cell. Samples were then harvested after 24 hr for determination of virus yield.

DISCUSSION

Our data provide direct evidence implicating a role for PKR in the regulation of IFN- α and IFN- β genes. We have demonstrated that suppression of PKR function results in impaired IFN induction at both the protein and mRNA levels. Our data further suggest that induction of IFN- α and IFN- β genes may rely differentially on PKR-dependent and PKR-independent signaling mechanisms. The induction of both IFN- α and IFN- β mRNA by EMCV was impaired in U937-AS1 and U937-M22 cells, and yet poly(I):poly(C) still induced IFN- β but not IFN- α mRNA. Given this, the induction of IFN- β by poly(I):poly(C) in these PKR-deficient cells cannot be easily explained as the result of residual PKR activity. It is possible, therefore, that alternative, PKR-independent pathways exist for dsRNA signaling. Consistent with this, protein-tyrosine kinases have been indirectly implicated in the induction of IFN-stimulated genes by dsRNA (20). However, we cannot rule out the possibility that residual, low levels of PKR remaining in the U937-AS1 and U937-M22 cells, while insufficient for mediating IFN- α induction, are sufficient for IFN- β induction by dsRNA. Analysis of mice with homozygous deletions for PKR will be useful for the characterization of PKR-independent signaling pathways by dsRNA. Furthermore, this study suggests that activation of PKR *in vivo* can occur in response to inducers other than dsRNA, since IFN induction by the combination of IFN- γ priming and subsequent LPS or PMA stimulation required functional PKR (Fig. 3A; Fig. 3C, lanes 7 and 8). Activation of PKR without dsRNA *in vitro* has been described using heparin and other polyanionic molecules, and PKR activation *in vivo* was observed following interleukin 3 deprivation of an interleukin 3-dependent murine cell line (21, 22).

Our results also provide evidence for the participation of PKR in mediating the antiviral actions of IFN- α and IFN- γ . While PKR has not been commonly considered as a mediator

of IFN- γ actions, the presence of a consensus IFN- γ responsive element, GAS, within the PKR promoter suggests that PKR may be regulated by IFN- γ (23). Consistent with this, our results demonstrated the induction of PKR protein levels by IFN- γ (Fig. 1B). Previous studies have linked several proteins, including the Mx, 2-5-oligoadenylate synthetase, and 2-5-oligoadenylate-dependent RNase proteins, to IFN- α -induced antiviral activities (24–26). In particular, stable expression of the human PKR gene in mouse cells confers partial resistance to EMCV (27). Also, in embryonic fibroblasts from mice deleted for the gene encoding the IFN-responsive transcription factor IRF-1, anti-EMCV activity by IFN- γ was even more impaired than the reduced IFN- α -mediated activity, characteristics similar to the PKR-deficient cells in this report (28). It is likely that the concerted actions of several genes, including PKR, contribute to the antiviral activities of IFN- α and IFN- γ . Interestingly, a tumor-suppressor function for PKR has been suggested from studies showing that a malignant transformation phenotype correlates with overexpression of dominant negative PKR proteins (29, 30). Since IFNs have direct anti-tumor and antiproliferative activities (31), it is possible that the IFN-related deficiencies resulting from loss of PKR activity noted here may represent mechanisms which contribute to a transformation process.

PKR has been suggested to be important for controlling viral replication. However, many viruses, including adenovirus, influenza virus, vaccinia virus, and human immunodeficiency virus, possess mechanisms for inactivating PKR function as means to evade the antiviral actions of the IFN system (32). We have shown that specific suppression of PKR in U-937 cells resulted in a profound inability to restrict EMCV replication and that this was due to the impairment of at least two biological functions, type I IFN expression and IFN-mediated antiviral responses. While it remains unclear which cellular proteins mediate these activities in pathways downstream from PKR, transcription factors including IRF-1, ATF-2/c-Jun, and the STAT family, already implicated with regulation of type I IFNs and IFN-stimulated genes, are possible substrates for PKR (3, 4, 33).

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